The Oxidation of Ethanol by Mammalian Liver

by

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Formal Declaration

I declare that I have written the dissertation to the University of Edinburgh for the degree of Doctor of Medicine; that it is based upon my own observations and that except as indicated in the thesis the data were collected, analysed and interpreted by me.

R.J.M. CORRALL.

THE OXIDATION OF ETHANOL BY MAMMALIAN LIVER

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PART 1. THEORETICAL BACKGROUND

Chapter 1. The Metabolism of Ethyl Alcohol

Ethanol is mostly metabolised in the liver via acetaldehyde to acetate. The latter is oxidised mainly in extrahepatic tissues. Following absorption and distribution of ethanol in man a constant rate of fall of blood levels is seen at concentrations below 100mg%. In some but not all studies involving chronic ethanol administration to man and animals it has been shown to induce its own metabolism.

Ethanol is thought to be oxidised to acetaldehyde by three enzymatic mechanisms. These are alcohol dehydrogenase (ADH), catalase and the microsomal ethanol oxidising system (MEOS). Alcohol dehydrogenase is a nicotine adenine dinucleotide (NAD)-linked cytoplasmic enzyme that transfers hydrogen to form NADH. There is convincing evidence that this enzyme oxidises ethanol *in vivo*. Catalase is able to oxidise ethanol "peroxidatically" to acetaldehyde and water in the presence of a H_2O_2-generating system *in vitro*. A microsomal ethanol oxidising system (MEOS) has been described *in vitro* similar to the cytochrome-P450 oxidase system responsible for drug metabolism in liver. Like that system it requires molecular oxygen and NADPH and transferred hydrogen appears in water. Reliable estimates of the relative contributions of catalase and MEOS to the overall metabolism of ethanol in liver are lacking. Problems associated with the use of inhibitors and of the unphysiological nature of disruptive subcellular preparations cast doubt on

present estimates. These two oxidative mechanisms may be more important at high concentrations of ethanol or with chronic administration.

Two major mechanisms of acetaldehyde oxidation in the hepatocyte have been described. These are a variety of flavoprotein enzymes and Racker's NAD-dependent aldehyde dehydrogenase enzyme. The flavoprotein enzymes require concentrations of acetaldehyde far higher than those found in vivo during ethanol oxidation. It therefore seems unlikely that these oxidise acetaldehyde in the hepatocyte during ethanol metabolism. Aldehyde dehydrogenase oxidises acetaldehyde to free acetate with the production of NADH. This abundant enzyme is active at physiological concentrations of acetaldehyde and is the mechanism generally considered to be responsible for its oxidation in liver.

Chapter 2. A Review of the Stereochemistry of Ethanol oxidation and of kinetic isotope effects

In ethanol two hydrogen atoms are attached to the ~~asymmetric~~ prochiral carbon atom C1. These are termed 1-(R) and 1-(S) in accordance with the notation of Cahn (1964). Paired enantiomers may be prepared in which one or other of these hydrogen atoms has been replaced by a heavy isotope of hydrogen. When alcohol is oxidised by alcohol dehydrogenase the 1-(R) hydrogen atom is selectively removed. This is transferred with an equally strict stereospecificity to the 4-(R) position of the pyridine ring of NAD in common with other A-type dehydrogenase enzymes.

Deuterium (D) and tritium (T) the heavy isotopes of hydrogen differ from protium only in the number of neutrons. The electronic structures of these three species are identical and qualitative chemical behaviour does not differ. However significant ~~differences in~~ ratios of the rate constants (KH/KD or KH/KT) of chemical reactions termed kinetic isotope effects may be observed. Primary kinetic effects result when a covalent bond binding the isotope is broken or formed. In secondary kinetic isotope effects this bond remains intact. The oxidation of isotopically substituted ethanol by liver alcohol dehydrogenase has been shown in different studies to exhibit a primary kinetic isotope effect (expressed as KH/KD) of between unity and 1.6. When the dissociation of NADH is no longer rate limiting a KH/KD of 4.2 has been observed with the equine liver enzyme.

Chapter 3. An Original Isotopic Method for the Quantification of Ethanol Metabolism in Tissue

The theory and development of an isotopic method enabling the quantification of pathways of ethanol oxidation in living tissue is described. The method depends upon the fact that hydrogen from ethanol is transferred to NADH by alcohol dehydrogenase and to water by MEOS and catalase. The different pathways are thus traced indirectly by following the fate of tritium transferred from ethanol labelled in the 1-(R) position. The incorporation of this tritium is compared with that removed from a labelled substrate known to donate hydrogen exclusively to the cytoplasmic NADH pool. The practical problems and theoretical assumptions involved in this method are discussed.

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Chapter 4. The Synthesis of the Enantiomorphs of [1-^{3}H] Ethanol

The preparation and purification of (S)-[1-^{3}H] ethanol and (R)-[1-^{3}H] ethanol is described. The putative structure was confirmed by counting acetaldehyde formed on oxidation of the 2 enantiomorphs with alcohol dehydrogenase.

Chapter 5. Stereospecificity of the Oxidation of Ethanol by Catalase

(R)-[1-^{3}H] ethanol and (S)-[1-^{3}H] ethanol were oxidised to acetaldehyde using bovine hepatic catalase. The acetaldehyde formed from the (S) but not the (R)-[1-^{3}H] ethanol contained tritium. The stereospecificity of catalase for alcohol is therefore the same as that of alcohol dehydrogenase.

Chapter 6. Stereospecificity of the Microsomal Ethanol Oxidising System

Microsomal suspensions from rat and monkey liver were prepared by three different methods and incubated with ethanol. [2-^{14}C] Ethanol in addition to (R)-[1-^{3}H] ethanol or (S)-[1-^{3}H] ethanol were added as tracers. Similar incubations were performed with the catalase-free MEOS preparation of Mezey *et al.* (1973). The ^{3}H:^{14}C ratio of acetaldehyde formed was compared with that of the labelled alcohol. In all experiments the results demonstrated a retention of the (S) hydrogen and a loss of the (R) hydrogen during the oxidation of ethanol to acetaldehyde by the microsomal systems. This indicates that MEOS in common with alcohol dehydrogenase and catalase selectively removes the (R) hydrogen during ethanol oxidation.

Chapter 7. Oxidation of the Enantiomorphs of [1-^{3}H] Ethanol by Rat Liver in vitro

Slices of rat liver were incubated with [1-^{14}C] ethanol and (R)-[1-^{3}H] ethanol or (S)-[1-^{3}H]ethanol. During the course of the incubation the ethanol in the flask containing the (S)-[1-^{3}H] ethanol but not that containing the (R) isomer was enriched with tritium. This is interpreted as an isotopic discrimination in the oxidation of [1-^{3}H] acetaldehyde. For an equivalent amount of tritium taken up from the medium from (S)-[1-^{3}H] ethanol much less isotope was incorporated into lactate than from the (R) isomer. This indicates that the (R) compared with the (S) hydrogen has a much greater access to the cytoplasmic NADH pool utilised in the reduction of pyruvate to lactate. It is concluded that the formation of NADH from acetaldehyde occurs primarily in a compartment other than the cytosol. Evidence from several sources is presented indicating that this compartment is mitochondrial thus invalidating (S)-[1-^{3}H] ethanol for use as a tritium donor to the cytoplasmic NAD pool.

Chapter 8. Determination of the Stereospecificity of Sorbitol Dehydrogenase

[2-^{3}H] Sorbitol was chosen as an alternative tritium donor to the NADH pool and experiments were therefore undertaken to determine the stereospecificity of sorbitol dehydrogenase. This was examined using 3 reference enzymes: lactate dehydrogenase (A type), aldehyde dehydrogenase (A type) and glucose dehydrogenase (B type). The transfer of labelled hydrogen indicated that sorbitol dehydrogenase

was an A-type enzyme. This fulfilled an absolute requirement for its use as the labelled hydrogen donor of the isotopic method outlined in chapter 3.

Chapter 9. Quantification of the Pathways of Ethanol Oxidation in Rat Liver

(R)-[1-^{3}H] Ethanol and [2-^{3}H]sorbitol have been used as tracers in experiments based on the method described in chapter 3. The incorporation of tritium from these two radio-chemicals has been examined in rat and monkey liver slices. The results indicate that in rat liver slices incubated at a medium ethanol concentration of 3 mg per ml non-alcohol dehydrogenase pathways are responsible for one tenth of ethanol oxidation. At a medium ethanol concentration of 1 mg per ml this contribution appeared negligible. In liver slices from rats maintained on an ethanol-rich diet the contribution of non-alcohol dehydrogenase pathways appeared quantitatively insignificant. However liver from this group of animals showed no increase in the amount of ethanol metabolised in vitro. In monkey liver slices at a medium ethanol concentration of 3 mg per ml the incorporation of tritium indicated that one tenth of ethanol oxidation proceeds via non-alcohol dehydrogenase pathways.

The possible developments of this isotopic method are discussed.

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Scientific Papers Published and Abstracts of Communications to Learned Societies

1. "Stereospecificity of the Oxidation of Ethanol by Catalase". Corrall, R.J.M., Rodman, H.M., Margolis, J. and Landau, B.R. J. Biol. Chem. (1974) 249, 3181.

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"In vino veritas"

Pliny the elder 23-79 A.D.

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PART I THEORETICAL BACKGROUND

CHAPTER 1. THE METABOLISM OF ETHYL ALCOHOL.

HISTORICAL INTRODUCTION

ALCOHOL METABOLISM IN THE INTACT ORGANISM

OXIDATION OF ETHANOL TO ACETALDEHYDE IN THE HEPATOCYTE: SUGGESTED PATHWAYS

THE OXIDATION OF ACETALDEHYDE TO ACETATE.

HISTORICAL INTRODUCTION

Ethyl alcohol is produced in plant tissues during anaerobic glycolysis, a process known as fermentation. Particularly high concentrations may be produced by those species such as yeasts that are capable of resisting its toxic action. Man's use of this simple molecule probably began in the palaeolithic era. In Egypt, Mesopotamia, Iran and the Far East cereal beers and wine have since 2000 BC been the usual excipients of therapeutic potions. Distillation proved a relatively simple means of increasing the strength of alcoholic beverages and in the nineteenth century modest wine stills (brûleries) were replaced by large scale distilleries. This burgeoning industry created grave social problems and in 1849 Huss coined the word "Alcoholism" to describe the effects of the abuse of distilled drink. The synthesis of ethanol was realised by Berthelot in 1860. This ushered in a period of active research into the physiology of ethanol which has continued unabated until the present day.

In 1872 Dupre demonstrated that alcohol recovered from the excretions represented an insignificant proportion of the amount administered. It was concluded on the basis of this and similar work that 95-98 per cent of ingested ethanol was totally oxidised to carbon dioxide and water. Atwater and Benedict (1902) and others showed that general metabolism was not increased by ethanol administration. The work of Strassman in 1891 showed that alcohol was capable of sparing protein as effectively as isocaloric amounts of fat indicating that alcohol could

be utilised by the organism as a foodstuff. Widmark's introduction of a micromethod for the determination of alcohol concentrations was a significant landmark. In the following decade the general quantification of alcohol absorption, distribution and elimination were worked out (Widmark, 1934).

ALCOHOL METABOLISM IN THE INTACT ORGANISM

Lundsgaard in 1938 was the first to show the pre-eminent role of the liver in ethanol metabolism. He demonstrated this by measuring the disappearance of ethanol in the eviscerated cat which exhibited only a small fraction of the normal rate of ethanol elimination. The importance of this organ in man has been confirmed by hepatic vein catheterisation (Lundquist, 1962; Tygstrup *et al.*, 1965). Larsen (1959) on the basis of infusion experiments has concluded that about one fifth of ethanol oxidation in man occurs in extra-hepatic tissue. The work of Forsander *et al.* (1960) in the rat suggests that skeletal muscle may be responsible for the bulk of this extra-hepatic metabolism. In the cat it seems that the kidneys may account for one third of this fraction (Larsen, 1963). Small amounts of ethanol are eliminated unchanged through the lungs and kidneys. At concentrations above 200 mg per 100 ml (44 mmol/L) this may exceed 10 per cent of the overall elimination rate.

Baron Justus von Leibig (1803-1873) assumed that ethanol was oxidised in living organisms via acetaldehyde, lactic, oxalic and formic acids to carbon dioxide.

Ample proof now exists implicating acetaldehyde as the intermediate in the oxidation of ethanol to acetate by the liver. In the first place all known pathways of quantitative importance produce acetaldehyde. In *in vitro* preparations it has been isolated as a reaction product (Lutwak-Man, 1938). Acetaldehyde is present in the blood during ethanol metabolism (in concentrations less than 0.2 mmol/L) and inhibition of its oxidation by tetraethylthiuramdisulphide (Antabuse) causes an increase in its concentration (Jacobson and Larsen, 1949; Wagner, 1957).

Studies using liver homogenates and perfused rat liver have shown ethanol to be almost quantitatively converted to acetate (Lundquist *et al.*, 1971; Williamson *et al.*, 1969). Acetate oxidation in liver is minimal due at least in part to an inhibition of the citric acid cycle by ethanol (Forsander *et al.*, 1965; Williamson *et al.*, 1969). Data obtained from liver slices (Forsander, 1966) and from studies in man (Lundquist *et al.*, 1962) indicate that oxidation of ethanol to acetate virtually monopolises the oxidative processes of liver. Furthermore Lundquist *et al.* (1962) were able to account for between 75 and 100 per cent of ethanol taken up by human liver as acetate in hepatic venous blood. Therefore it would appear that most of the acetate resulting from acetaldehyde oxidation is metabolised outside the liver. The biochemical fate of acetate is well understood and will not be discussed.

The early work in man of Widmark (1934) and of others including Haggard and Greenberg (1934), Schonheyder (1942)

and Lundquist (1958) has shown a constant rate of fall of blood ethanol in man following absorption and distribution. Widmark calculated correctly from the slope of the decline in blood alcohol levels that an average individual can oxidise about 1 gram of ethanol per hour per 10 Kg body weight. The constant rate of fall may only be accepted for the concentration range of 4 to 20 mmol/L (20 to 100 mg per 100 ml) to which observations have been confined. From work in man (Newman, 1949; Mendelson, 1968) and animals (Ewing, 1940; Eggleton, 1940) however it seems that metabolism at high blood concentrations may occur at a greater rate than expected from the rate constant obtained below 20 mmol/L. The contribution of increased urinary and respiratory losses to this has not been satisfactorily examined. However the possibility that this increase in elimination rate represents an alternative means of ethanol oxidation by the hepatocyte must be seriously entertained.

Chronic alcoholics have been shown to metabolise alcohol more rapidly than normal individuals (Kater et al., 1969; Ugarte, 1970). Experimentally the chronic administration of ethanol to rats (von Wartburg and Rothlisberger, 1961; Dajani and Orten, 1962; Hawkins et al., 1966; Videla and Israel, 1970; Mezey, 1972) to primates (Pieper and Skeen, 1973) and to man (Isbell et al., 1955; Mendelson et al., 1965; Kater et al., 1969; Mezey and Tobon, 1971) has induced an increase in the rate of ethanol metabolism. However other workers have failed to confirm this finding in rats (Kinard and Hoy,

1960; Segoria-Riquelone et al., 1962; Greenberger et al., 1965; Majchrowicz et al., 1968) and in man (Clark and Senior, 1968).

OXIDATION OF ETHANOL TO ACETALDEHYDE IN THE HEPATOCYTE: SUGGESTED PATHWAYS

Although conjugation of ethanol with sulphate (Bostrom and Vestermark, 1960) and as glucuronide (Kamil et al., 1952) and fatty acid ester formation (Newsome and Rattray, 1965) have been described these routes are quantitatively insignificant in the overall metabolism of ethanol. To explain the oxidation of ethanol to acetaldehyde three principal reaction mechanisms have been postulated. These are Alcohol Dehydrogenase (A.D.H.), Catalase and the Microsomal Ethanol Oxidising System (M.E.O.S.). The underlying chemical reactions are shown in Fig. 1.1.

1. Alcohol Dehydrogenase

Alcohol Dehydrogenase, an enzyme of the cell cytoplasm catalyses the oxidation of ethanol to acetaldehyde (Fig. 1.1,A). The requirement for nicotinamide adenine dinucleotide (N.A.D.) was established by Lutwach-Mann in 1938. The enzyme was first prepared in crystalline form by Bonnichsen and Wassen (1948). The equilibrium position is very unfavourable for the oxidation of ethanol:

$$\frac{[NADH]\ [H^+]\ [CH_3CHO]}{[NAD^+]\ [CH_3CH_2OH]} = 8 \times 10^{-12}.$$

This value was obtained by Backlin (1958) at 20^{o}. The reaction is driven to the right in vivo by the low ratio

Fig. 1.1. PATHWAYS OF ETHANOL OXIDATION TO ACETALDEHYDE IN THE LIVER

A. ALCOHOL DEHYDROGENASE (A.D.H.)

$$CH_3.CH_2.OH + N.A.D^+ \rightleftharpoons CH_3.CHO + NADH + H^+$$

B. CATALASE

$$CH_3.CH_2.OH + H_2O_2 \longrightarrow CH_3.CHO + 2H_2O$$

C. MICROSOMAL ETHANOL OXIDISING SYSTEM (M.E.O.S.)

$$CH_3.CH_2.OH + NADPH + H^+ + O_2 \longrightarrow CH_3.CHO + NADP^+ + 2H_2O$$

of free NADH to NAD and by the very effective removal of acetaldehyde.

Pioneering work (Theorell and Bonichsen, 1951; Theorell and Chance, 1951) established the generally accepted reaction sequence for the oxidation of ethanol as follows*:

1. $NAD^+ + E \rightleftharpoons E - NAD^+$
2. $E - NAD^+ + C_2H_5OH \rightleftharpoons H^+ + CH_3.CHO + E - NADH$
3. $E - NADH \rightleftharpoons E + NADH$

E signifies one polypeptide chain capable of binding one molecule of NAD^+ (v. infra.). The overall equilibrium constant calculated from the kinetic constants of the above three reactions approximates closely to the value determined directly (Theorell and McKinley-McKee, 1961).

The human enzyme has a molecular weight of about 87,000 (von Wartburg et al., 1964) and contains two functional atoms of zinc. (Blair and Vallee, 1966). Human A.D.H. has a Km for ethanol of 1.2 mmol/L (5.5 mg per 100 ml) at pH 8.8 (von Wartburg et al., 1964). It seems likely that the enzyme consists of two similar polypeptide chains each containing a reactive -SH group in a cysteine residue (Harris, 1964). The liver enzyme (unlike that obtained from yeast) exhibits a relative specificity oxidising a broad group of primary and secondary alcohols. (Theorell, 1965). An atypical form of human A.D.H. has been described (v. Wartburg and Schurch, 1968). Although in vitro at a

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*Step 3 is thought to be rate limiting in vitro - see page 2.7.

physiological pH it seems more active than the normal variety, individuals possessing this isoenzyme do not metabolise ethanol at a greater than normal rate (v. Wartburg and Schurch, 1968).

Human and equine livers contain sufficient A.D.H. to explain the elimination rate observed in these species (Lester and Keokowsky, 1967; v. Wartburg et al., 1964). Furthermore strong but indirect evidence that supports the participation of A.D.H. in ethanol metabolism comes from two additional sources. Firstly estimations of the apparent Km from blood ethanol disappearance curves give values similar to those obtained with the pure enzyme (Lundquist and Wolthers, 1958; Maker and Mannering, 1970). Secondly ethanol metabolism is associated with redox changes (decrease in the ratio of free NAD^+: free NADH in cytosol and mitochondrion) that would result from oxidation via a NAD-linked dehydrogenase (Forsander, 1970). However a poor correlation exists between measured ADH activity and the rate of ethanol metabolism under varying conditions (Wallgren and Barry, 1970). Present evidence suggests strongly that the availability of NAD (determined by the rate of reoxidation of NADH) is the rate limiting factor in the metabolism of ethanol by ADH in vivo (Forsander, 1971). The mitochondrial membrane is impermeable to the coenzyme and a carrier system must be involved in transporting the hydrogen of NADH to its site of oxidation. Current evidence favours a shuttle system involving aspartate and malate (Williamson et al., 1974).

2. Catalase

Catalase activity was originally described in 1911 by Thénoud in a tissue extract capable of decomposing hydrogen peroxide with the evolution of oxygen (Summer, 1941). The enzyme is widespread in nature with the striking exception of obligate anaerobes. Beef liver catalase has a molecular weight of 248,000 and contains four haem groups of protoporphyrin IX (Fe^{+++}) per enzyme molecule. The breakdown of hydrogen peroxide with oxygen production has been referred to as its "catalatic" mode. This involves the formation of an intermediate, Compound I (Chance, 1947):

$$\text{Cat.} + H_2O_2 \rightleftharpoons \text{Cat} - H_2O_2 \text{ (Compound I)}$$

$$\text{Cat} - H_2O_2 + H_2O_2 \longrightarrow \text{Cat} + 2H_2O + O_2$$

The second molecule of hydrogen peroxide functions as hydrogen donor in this "catalatic" reaction. With other hydrogen donors including ethanol the catalase-H_2O_2 intermediate (Compound I) is also decomposed to catalase and water coupled with the oxidation of the hydrogen donor. Thus ethanol may be oxidised to acetaldehyde:

$$\text{Cat-}H_2O_2 + CH_3CH_2OH \longrightarrow \text{Cat} + 2H_2O + CH_3CHO.$$

The overall reaction for ethanol is given in Fig. 1.1,B. This is called the "peroxidatic" reaction and was first described in 1945 by Keilin and Hartree.

A possible role for catalase in ethanol oxidation *in vivo* was first suggested by Keilin and Hartree in 1936. At least two coupled peroxidatic reactions involving aldehyde oxidase (Keilin and Hartree, 1945) and cysteine (McGuire, 1965) have been described *in vitro*. However it

seems most unlikely on theoretical grounds that either of these could operate *in vivo*. Several studies have been performed with the catalase inhibitor 3-amino-1,2,4-triazole. Using this compound no reduction in ethanol metabolism rate could be shown (Nelson *et al.*, 1957). Furthermore Lundquist *et al.*, (1963) failed to observe any significant effect with this inhibitor in rat liver homogenates. Videla and Israel (1971) using rat liver slices were able to show that aminotriazole could significantly inhibit the increase in ethanol metabolism induced by chronic ethanol treatment. No effect was seen in liver slices from rats not previously exposed to ethanol.

A detailed phenomenological study from Chance's laboratory on the oxidation of ethanol by catalase has recently been published (Oshino *et al.*, 1973). This has questioned the interpretations of previous work on the role of catalase in ethanol metabolism. In particular it has questioned the validity of work using catalase inhibitors. Peroxidatic ethanol oxidation by rat liver catalase was studied quantitatively with respect to the rate of H_2O_2 generation, catalase haem concentration and the steady state concentration of Compound I. The phenomena observed could be simplified by invoking the ratio: H_2O_2 generation rate (expressed as μmol/L per minute) to catalase haem concentration (expressed as μmol/L). This will be referred to as the H:C ratio. It was found that at H:C ratios below 60/min oxidation was almost completely peroxidatic and changes in catalase concentration did not affect the rate of ethanol oxidation which was determined by the rate

of H_2O_2 generation. When the H:C ratio exceeded 200/min a significant decrease in ethanol oxidation occurred due to an increase in the proportion of catalatic decomposition of H_2O_2. In addition by altering the concentration of ethanol it was shown that the proportion of peroxidatic oxidation was a function of the ethanol concentration increasing with higher ethanol concentrations. In other words to quantitate the oxidation of ethanol by catalase in tissues a knowledge of the H:C ratio is mandatory. The oxidation of ethanol by catalase is clearly very complex depending on the contribution of "catalatic" and "peroxidatic" reactions to the overall decomposition of H_2O_2. Phenomena resulting from this include invariance to the catalase concentration (under certain conditions), variability of inhibitor effect and variability of the apparent Km of ethanol.

From these observations it is evident that the negative results with aminotriazole quoted above do not necessarily rule out a participation of catalase in ethanol oxidation. At 1mM* ethanol concentration and H:C ratio of 0.4/min to achieve a 50% reduction in ethanol oxidation rate it would be necessary to inactivate catalase by 98.7% if the H_2O_2 generation rate did not change. Clearly unless the H_2O_2 generation rate and catalase haem concentrations are known in an experimental preparation it is not possible to draw meaningful conclusions from the use of such inhibitors. Parenthetically it may be added that the inhibition observed by Vidda and Israel does not a priori prove a role for catalase because

*M is used to signify molar i.e. gram-molecules per litre.

of the possibility of a lack of specificity of inhibition with aminotriazole.

At present until more data becomes available on the rate of H_2O_2 generation in liver it is not possible to make a definitive statement regarding the role of catalase in ethanol metabolism. However the demonstration by Chance et al., 1974 (quoted by Oshino et al., 1973) and Lindros et al., 1974 that Compound I levels in the perfused liver respond to exogenous alcohol suggests strongly that catalase may indeed have a role in alcohol metabolism.

3. Microsomal Ethanol Oxidising System (M.E.O.S.)

Mueller and Miller (1949, 1953) provided the first description of a hepatic microsomal* system capable of metabolising foreign compounds. Brodie and co-workers in 1955 examined a similar system capable of metabolising many drugs that required NADPH and Mg^{++} for full activity. Posner et al. (1961) employed $^{18}O_2$ and $H_2{}^{18}O$ and showed that oxygen utilised in hydroxylation was derived from molecular oxygen rather than water. This oxidising activity with requirements for NADPH and O_2 has been termed the "mixed function oxidase system" by Mason (1957) and the monooxygenase system by Hayaishi (1964).

The overall reaction of this system in which a hypothetical compound RH is hydroxylated is as follows:

$$RH + NADPH + O_2 + H^+ \longrightarrow ROH + NADP^+ + H_2O$$

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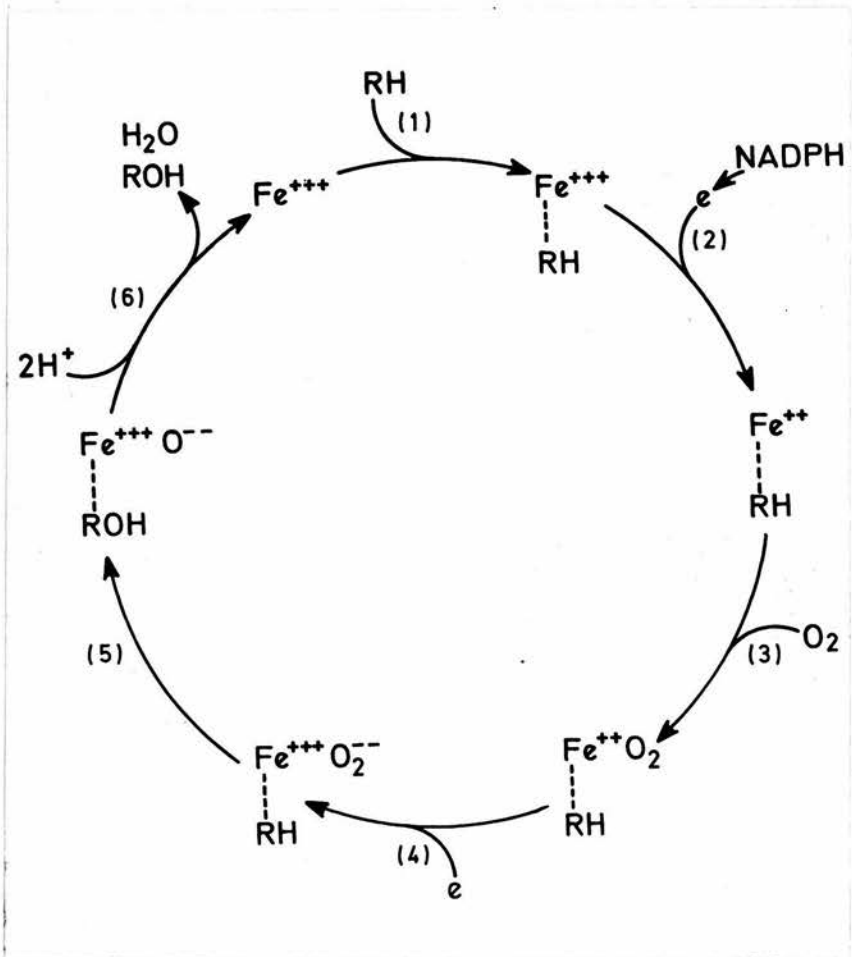
*Microsomes are the in vitro equivalent of the smooth endoplasmic reticulum (S.E.R.) in the intact cell.

Lu and Coon (1969) have shown that this monooxygenase system contains three important components. These are cytochrome P-450, NADPH-cytochrome P-450 reductase and a heat-stable lipophilic component. Cytochrome-P450 is so named because of a prominent absorption band at 450nm resulting from combination of the reduced (ferrous) form with carbon monoxide. This haemoprotein acts as the terminal oxidase of the monooxygenase system. Its concentration in liver increases with the non-specific induction that results from chronic exposure to many xenobiotics. NADPH-cytochrome P450, reductase (syn. NADPH-cytochrome c reductase) is a flavine-adenine-dinucleotide (FAD) containing enzyme that transfers reducing equivalents from NADPH to cytochrome P450. The heat stable lipophilic component is replaceable by phospholipid particularly phosphatidyl choline.

The hypothetical reaction sequence modified after Ullrich (1972) is shown in Fig. 1.2. The initial reaction (1) consists of the binding of substrate to the oxidised (ferric) form of cytochrome P-450. This combination is then reduced by NADPH-cytochrome P450 reductase (2) by one electron transfer. This reduction is considered rate limiting for the overall monooxygenation pathway (Davies et al., 1969; Gigon et al., 1969; Gillette and Gram, 1969). The next step involves interaction with molecular oxygen (3) and subsequent acceptance of a second electron (4) with the formation of an unidentified active oxygen-cytochrome-substrate complex. The source of this second electron is not known. It has been suggested that it may

Fig. 1.2. HYPOTHETICAL REACTION SEQUENCE OF THE CYTOCHROME-P450 MICROSOMAL OXIDATION SYSTEM



originate from NADH via cytochrome b_5 and the enzyme NADH-cytochrome b_5 reductase (Cohen and Estabrook, 1971; Hildebrant and Estabrook, 1971). However the evidence is far from convincing (Gillette et al., 1972). Following transfer within the complex of an oxygen atom (5) the complex dissociates after the uptake of protons (6) yielding oxidised substrate, water and oxidised cytochrome P450. The exact mechanism of P-450-linked monooxygenation is still sub judice and it is possible that alternative mechanisms may exist. For example there is evidence suggesting that the superoxide radical (O_2^-) may be involved in the reaction sequence (Strobel and Coon, 1971). Of interest also is the recent demonstration that P-450 exhibits peroxidatic activity (Hyrcray and O'Brian, 1971).

The spectral changes observed upon addition of a great variety of compounds to liver microsomes have been classified into three groups (Schenkman et al., 1967). Type I typified by hexobarbital has a peak at 390nm and a trough at 420nm. This spectral change is associated with known substrates for oxidation and probably represents combination of substrate with cytochrome P-450. Type II compounds such as aniline produce a trough near 390nm and a peak at 425-435nm. These chemicals are basic amines and generally do not serve as substrates. The spectral change is thought to represent interaction of a haem iron with the basic nitrogen of the added compound. The third change has been called "modified type II" and is described in a later section.

Orme-Johnstone and Ziegler in 1965 studied the oxidation of ethanol to acetaldehyde in microsomal suspensions of pig, rat and rabbit liver. This system was more active in oxidising methanol than ethanol and was not inhibited by carbon monoxide. It was however inhibited by cyanide and azide. Lieber and DiCarli (1968, 1970) have described in detail a similar system (M.E.O.S.). It differed from that of Orme-Johnstone and Ziegler in being sensitive to carbon monoxide and in oxidising ethanol at a higher rate than methanol. In contradistinction to alcohol dehydrogenase its optimum pH is 7.2 to 7.4 (c.f. 10.8) and its Km is 8mM (c.f. 2mM). The proposed overall reaction of ethanol oxidation via M.E.O.S. is given in Fig. 1.1(C). Lieber has equated this oxidation system with the P450-linked monooxygenase system responsible for the oxidation of xenobiotics. The same author (Lieber, 1972) has calculated (making allowance for P-450 loss during microsomal preparation) that M.E.O.S. accounts for 20 to 25% of ethanol metabolism in the non-induced animal. Whether observed M.E.O.S. activity results from the P-450-linked monooxygenase system responsible for drug metabolism is an area of great controversy. An alternative widely held view is that the observed oxidation of ethanol is an _in vitro_ artefact resulting from the contamination of microsomes by catalase. The evidence both for and against the existence of M.E.O.S. as a unique mode of ethanol oxidation involving cytochrome P-450 _in vivo_ can be summarised under the following headings:

(i) Inductive and inhibitory phenomena with alcohol and drugs. Lieber (1970) has demonstrated that chronic administration of phenobarbitone or ethanol may cause an increase in the constituents of the hepatic smooth endoplasmic reticulum (S.E.R.) including cytochrome P-450. This was associated with an increase in M.E.O.S. activity. From the same laboratory (Lieber and DiCarli, 1970) the feeding of an ethanol-containing diet for 24 days to rats was reported to result in a higher rate of clearance of ethanol from blood and a higher M.E.O.S. activity. A.D.H. and catalase activities did not change. Rubin et al. (1970, 1970a) have shown that ethanol in vitro in concentrations comparable to those found in the blood of inebriated individuals can cause an inhibition of drug metabolism in microsomes. The above observations are consistent with the metabolism of ethanol via a microsomal oxidation system responsible for the metabolism of xenobiotics. They also conveniently suggest explanations for the resistance of the alcoholic to drugs and for the potentiation of drugs taken concurrently with ethanol. However Porta et al. (1969, 1970) although able to produce an increase in S.E.R. after two weeks of ethanol feeding could not demonstrate an increase at four and sixteen weeks. Furthermore these workers were able to increase the S.E.R. by replacing ethanol by an isocaloric amount of fat. In addition several workers (Klaassen, 1969; Khanna and Kalant, 1970; Mezey, 1971) using a variety of inducers of drug metabolism including phenobarbital have been unable to show any effect on the rate of ethanol metabolism. Finally Khanna et al. (1972) were able to

induce M.E.O.S. and increase metabolic clearance of alcohol by chronic ethanol feeding. However merely by changing the protein content of the diet they achieved an increased metabolic clearance of ethanol without any corresponding increase in M.E.O.S. activity.

(ii) Spectral changes. The addition of ethanol to hepatic microsomes results in a modified type II spectrum with a trough at 390 to 394 nm and a peak at 415 to 420 nm (Rubin et al., 1971). This would seem to indicate a combination of ethanol with microsomal haemoprotein and thereby provide evidence (albeit indirect) for its P-450-linked monooxygenase oxidation. However compounds that elicit the modified type II spectral change are a heterogenous group. They include steroids, drugs, alkaloids, tryptophane as well as several alcohols. Some appear to serve as substrates for the mixed function oxidase system while others do not. Furthermore Imai and Sato (1967) have pointed out that the concentrations of alcohols inducing spectral changes are of the same order of magnitude as those known to cause conformational changes in proteins. In addition the relative intensity of spectral changes: isoamyl > isobutyl > isopropyl > ethyl > methyl is in the same order as is associated with a tendency to induce conformational change by a disruption of hydrophobic bonds. Recently Comai and Gaylor (1975) have described three spectrally distinguishable forms of cytochrome P-450 in rat liver microsomes. One of these, type I was increased by ethanol administration and exhibited the highest affinity for cyanide.

(iii) Studies using Enzymatic inhibitors. The demonstration that MEOS activity is not inhibited by concentrations of pyrazole that effectively inhibit ADH supports the contention that MEOS may metabolise ethanol independently of ADH *in vivo* (Khanna *et al.*, 1970; Lieber and DeCarli, 1970). Studies with SKF-525A (a potent inhibitor of the P-450 monooxygenase system) both *in vitro* (Khanna *et al.*, 1970; Lieber and DeCarli, 1970) and *in vivo* (Telphly *et al.*, 1969; Khanna and Kalant, 1970) have failed to show any reduction in the rate of oxidation of alcohol. The metabolism of some drugs however - especially those that do not induce a type I spectral change - is relatively insensitive to the action of that inhibitor (Anders, 1971). A reduction in MEOS activity has been reported to result from the catalase inhibitor aminotriazole (Roach *et al.*, 1969; Isselbacher and Carter, 1970; Khanna *et al.*, 1970). Lieber and DeCarli (1970) however found that this inhibitor at a concentration inhibiting catalase activity by 90-95% reduced that of MEOS by only 35 to 50%. The same authors found that cyanide (0.1mM) inhibited MEOS only slightly but resulted in virtually complete inhibition of catalase. However Roach *et al.*, (1969) using cyanide in a concentration of 1 mmol/L demonstrated complete inhibition of MEOS despite retention of drug-metabolising activity. MEOS activity is partially inhibited by carbon monoxide but the effect is less striking than for other microsomal monooxygenase activity and the $CO:O_2$ ratio required is higher (Lieber and DeCarli, 1970). The peculiar problems associated with the interpretation of data using enzyme

inhibitors are discussed on pages 1.10 and 1.21 of this chapter.

(iv) Cytochrome P-450 isolation and re-combination experiments. Recently Teschke et al. (1972) and Mezey et al. (1973) have effected a separation of MEOS activity from contaminating catalase and ADH by using DEAE-cellulose chromatography with solubilised microsomes. Absence of catalase was verified using an oxygen electrode. The MEOS-rich fraction contained phospholipids, NADPH-cytochrome P-450 reductase and cytochrome P-450. This demonstration by two independent groups of workers appears to give strong support for the presence of a P-450-linked monooxygenase system capable of oxidising ethanol. However it is possible that catalase in amounts insufficient to be detected by the oxygen electrode could be responsible for the small amount of ethanol oxidation observed by these workers. Furthermore Thurman and Scholz (1973) have been unable to demonstrate ethanol oxidation in a reconstituted catalase - free mixed function oxidase system capable of metabolising benzamphetamine.

(v) Miscellaneous Data. Evidence quoted in support of the participation of catalase in MEOS activity is the ability of microsomal suspensions to oxidise ethanol when NADPH is replaced by a hydrogen peroxide generating system (Roach et al., 1969; Isselbacher and Carter, 1970) or barium peroxide (Khanna et al., 1970). The evanescence of MEOS activity in vitro even with removal of acetaldehyde (Khanna et al., 1970; Lieber and DeCarli, 1970) casts doubt on its existence as a significant pathway in vivo.

Furthermore estimation of the contribution of MEOS to total ethanol metabolism may be excessive if based on such figures of maximal but transient activity. Finally the study of Khanna *et al.* (1971) in which carbon tetrachloride administration caused a dramatic reduction in MEOS activity without any quantitative effect on ethanol oxidation in rats further questions the existence of the so-called MEOS pathway *in vivo*.

From the data presented above there are three possible conclusions that may be drawn. Firstly that so called MEOS activity is an artefact of an *in vitro* preparation resulting from catalase and a H_2O_2 generating system and that a mixed function oxidase system capable of oxidising ethanol does not exist in the intact cell. Certainly microsomal suspensions are capable of forming hydrogen peroxide (Thomson *et al.*, 1972) and microsomes as conventionally prepared do contain significant quantities of catalase (Teschke *et al.*, 1972; Mezey *et al.*, 1973). Secondly that as suggested by Lieber a system located in the SER and involving cytochrome P-450 but not catalase is responsible for the oxidation of ethanol in liver. It is possible that the underlying mechanism is peroxidatic (Hrycay and O'Brian, 1971). Thirdly, that a MEOS does exist and involves the participation of catalase *in vivo*. This seems unlikely because catalase is thought to be confined to the peroxisomes in the cell (de Duve and Bandhuin, 1966) although in homogenates it is found tightly bound to microsomal membranes (Roach *et al.*, 1969; Carter and Isselbacher, 1971).

I do not feel that it is possible at present to distinguish between these three possibilities on the basis of published evidence. It is conceivable that a stoichiometric study of MEOS activity with quantitation of H_2O_2 production rates or alternatively that studies using $^{18}O_2$ and $H_2{}^{18}O_2$ (introduced by the method of Laser, 1955) could give useful information about the mechanism of oxidation by microsomes *in vitro*. However one would still be left with the question of how well such preparations reflect the true biochemical situation in the intact hepatocyte.

The Relative Contribution of Different Pathways to Ethanol Metabolism

The biochemistry of the three putative routes of ethanol oxidation to acetaldehyde has been summarised. The contribution of each to the total metabolism of ethanol is difficult or impossible to assess for two major reasons: (a) The *in vitro* methods used are extremely artificial. Thus the concentration of enzymes, substrates, coenzymes and other co-factors employed may differ greatly from those present inside the cell. In addition the pH may differ significantly e.g. ADH activity is customarily measured at an unphysiologically high pH. The partial pressure of oxygen is increased more than ten fold when 95% O_2 is employed *in vitro*. Furthermore homogenisation procedures disrupt the sub-cellular architecture resulting in a highly abnormal mixture of enzymes, coenzymes ~~and~~

and substrates not present *in vivo*. Because of these factors estimates of the contribution of particular pathways to the total liver metabolism must be highly suspect. (b) The large number of studies using enzyme inhibitors that have arrived at quite disparate conclusions testifies to the limited value of these compounds in the investigation of metabolic pathways. Certainly it may be said of these investigative tools that specificity diminishes with familiarity. Thus aminotriazole and pyrazole can both inhibit a variety of microsomal enzyme activities (Kato, 1967; Lieber *et al.*, 1970). In addition azide is not a specific inhibitor of catalase (Lieber *et al.*, 1970) and cholate a presumed specific inhibitor of NADPH oxidase (implicated in microsomal H_2O_2 formation) is a non-specific disruptor of microsomal function. A further problem with regard to inhibitors is the location of the rate limiting step in different pathways. If an inhibitor does not act at this step the results of inhibition are difficult to predict and may be minimal. The rate limiting step for the ADH pathway probably relates to the disposal of reducing equivalents on NADH. The rate of peroxidatic oxidation by catalase is probably limited by the rate of H_2O_2 generation. This makes the effect on overall rate of enzymatic inhibition difficult to predict for these two pathways. Additional problems are encountered with catalase inhibitors (v. supra.) that makes data obtained difficult or impossible to analyse without a knowledge of the H_2O_2 generation rate and catalase haem concentration.

From the present evidence therefore it does not seem possible to provide an accurate quantitative estimate of the different pathways responsible for the oxidation of ethanol to its aldehyde. Alcohol Dehydrogenase is clearly involved and there is sufficient enzyme in the liver to explain the overall rate of ethanol metabolism. Catalase and/or MEOS may be operating *in vivo* and could possibly assume a greater importance at higher ethanol concentrations or following chronic ethanol intake. In chapter 3 an original method using specifically labelled ethanol will be outlined that has been developed to quantify these pathways. In part 2 of this thesis the practical application of this method will be described.

THE OXIDATION OF ACETALDEHYDE TO ACETATE

Acetaldehyde oxidation occurs principally in the liver. This conclusion is supported indirectly by the data of Lundquist (1962) that 75 to 100% of ethanol uptake appears as acetate in hepatic venous blood. Furthermore in cats evisceration causes a 4 to 5 fold delay in elimination of exogenous acetaldehyde (Lubin and Westerfield, 1945) and perfused rabbit livers can metabolise up to 80 per cent of that found in intact animals (Hald *et al.*, 1949).

Possible Enzymatic Mechanisms in the Hepatocyte

By far the largest part of acetaldehyde is oxidised to acetate but other fates have been described. Thus formation of acetoin (Stotz *et al.*, 1944; Berry and Stotz, 1954) of 5-hydroxy-4-keto,-n-hexanoic acid

(Westerfield, 1969) and of threonine (Gilbert, 1957; Karasek and Greenberg, 1957) has been demonstrated but none of these reactions are considered quantitatively important. The two possible enzymatic methods responsible for oxidation of acetaldehyde to acetate will be described below.

1. Flavoprotein Enzymes. Many such enzymes capable of oxidising aldehydes and other compounds such as quinine, purines and pteridines have been isolated from liver. A flavoprotein which oxidises acetaldehyde but not xanthine (thus differentiatingit from xanthine oxidase) has been prepared from rabbit liver. (Rajagopalan et al., 1962; Rajagopalan and Handler, 1964 and 1964a). This enzyme containing iron, molybdenum and FAD is potentially capable of oxidising acetaldehyde aerobically in vivo though the exact mechanism is not known. However such enzymes require concentrations of acetaldehyde far higher than those found in vivo after ethanol administration (Lundquist et al., 1962). Furthermore acetaldehyde oxidation is not affected by molybdenum deficiency (Kichert and Westerfield, 1957). It seems most unlikely therefore that such enzymes are of importance in vivo.

2. NAD-Dependent Aldehyde Dehydrogenases. In 1949 Racker described the preparation from bovine liver of an aldehyde dehydrogenase free from contamination by alcohol dehydrogenase. This enzyme capable of oxidising a wide variety of aliphatic and aromatic aldehydes catalyses the following reaction:

$$NAD^+ + CH_3CHO + H_2O \rightleftharpoons CH_3COOH + NADH + H^+$$

The reaction product was identified as free acetate and not the coenzyme A derivative as observed with some bacterial enzymes. The Km for acetaldehyde is extremely low at 10^{-6} mol/L (Racker, 1949) or 10^{-9} mol/L Buettner, 1965). An enzyme similar in specificity and Km value has been isolated from human liver (Kraemar and Deitrich, 1968; Blair and Bodley, 1969). As in other mammals the enzyme was considered mainly cytoplasmic. Buettner (1965) has estimated that in the rat aldehyde dehydrogenase is present in 4 to 5 times higher activity than ADH. This high activity and low Km value make it eminently suitable to maintain the low levels of acetaldehyde present during alcohol metabolism. Until recently it was generally accepted that acetaldehyde oxidation occurred via aldehyde dehydrogenase in the cytoplasm. However this view will be discussed further in chapter 7 in the light of my experimental data and in that of recent data from several laboratories.

CHAPTER 2: A REVIEW OF THE STEREOCHEMISTRY OF ETHANOL OXIDATION AND OF KINETIC ISOTOPE EFFECTS

1. STEREOCHEMICAL ASPECTS OF THE ENZYMATIC OXIDATION OF ETHANOL.

INTRODUCTION

THE STEREOCHEMISTRY OF ETHYL ALCOHOL

THE STEREOSPECIFICITY OF ETHANOL OXIDATION BY ALCOHOL DEHYDROGENASE

2. KINETIC ISOTOPE EFFECTS.

INTRODUCTION

PRIMARY AND SECONDARY KINETIC ISOTOPE EFFECTS

HYDROGEN ISOTOPE EFFECTS IN THE OXIDATION OF ETHANOL

CHAPTER 2

A REVIEW OF THE STEREOCHEMISTRY OF ETHANOL OXIDATION AND OF KINETIC ISOTOPE EFFECTS

1. STEREOCHEMICAL ASPECTS OF THE ENZYMATIC OXIDATION OF ETHANOL

Introduction

In 1848 Louis Pasteur manually separated two morphologically dissymmetric species of the hemihedral crystals of sodium ammonium tartrate. He subsequently showed that aqueous solutions of these crystals were optically active, the specific rotations being equal in magnitude and opposite in sign. He concluded correctly that this optical activity must be due to a dissymmetric assembly of the component atoms in tartaric acid. His demonstration of fermentation by a species of yeast of only the dextrorotatory form constituted the first stereospecific enzyme catalysis (Pasteur, 1858). These brilliant observations were followed by the theory of the tetrahedral structure of the carbon atom in 1874 by Van't Hoff (Cohen, 1912) which gave a structural basis to explain the dissymmetry postulated by Pasteur. Hand in hand with the development of stereochemistry there developed an increasing knowledge of the asymmetric nature of enzyme catalysis. The oxidation of ethanol by alcohol dehydrogenase has proved no exception to this rule of asymmetric stereospecificity.

The Stereochemistry of Ethyl Alcohol

Fig. 2.1 depicts the molecule of ethyl alcohol as conventionally drawn (A) and in a stereochemical

representation of its tetrahedral structure (B). Photographs of a molecular model of the same compound as viewed from in front (i.e. as in Fig. 2.1 A & B) from behind and from above are shown in Fig. 2.1 C, D and E respectively. The pro-chiral carbon atom C-1 (otherwise termed the meso, methylene or asymmetric carbon atom) has four substituents. These are a methyl and hydroxyl group and two hydrogen atoms labelled (R) and (S) in accordance with the notation of Cahn (1964). Derivatives may be prepared (see Chapter 4) in which either R or S hydrogen is replaced by a heavy isotope of hydrogen (Fig. 2.2 A). These stereoisomers are termed enantiomers (syn. enantiomorphs) because of their relationship to each other as non-superimposable mirror images.

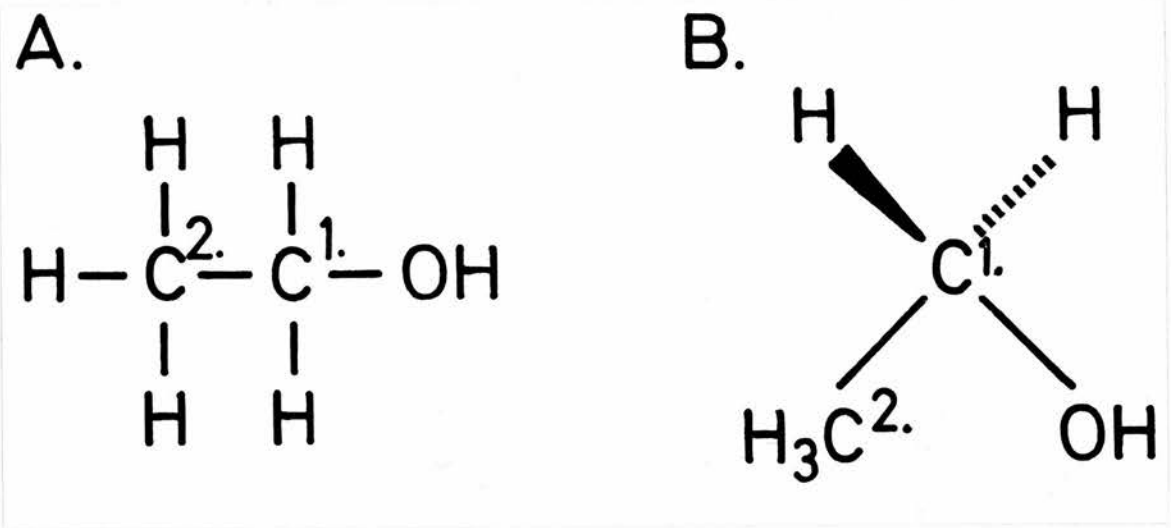
The Stereospecificity of Ethanol Oxidation by Alcohol Dehydrogenase

Vennesland and her co-workers (see Vennesland and Westheimer, 1954) have shown in an elegant series of experiments that alcohol dehydrogenase demonstrates strict stereospecificity in removing exclusively the (R) hydrogen during oxidation of ethanol. In the reverse direction when acetaldehyde is reduced to alcohol the hydrogen is added in the same position. The absolute configuration of these two enantiomers was confirmed by Lemieux and Howard (1963).

Hydrogen is transferred* to position 4 of the pyridine ring of NAD during oxidation by NAD-linked dehydrogenase

*The hydride ion i.e. the hydrogen atom with two electrons is thought to be the molecular species involved in this direct hydrogen transfer (Colowick et al., 1966).

Fig. 2.1A,B. ETHYL ALCOHOL

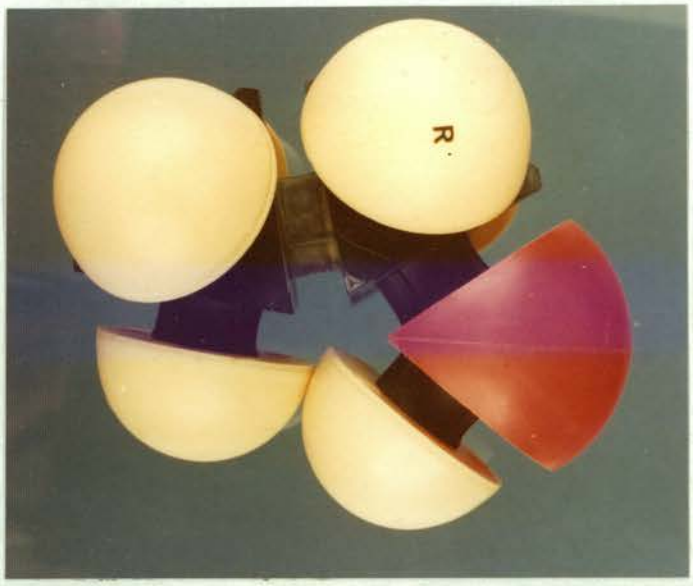


A. Chemical structure with numbering of carbon atoms.

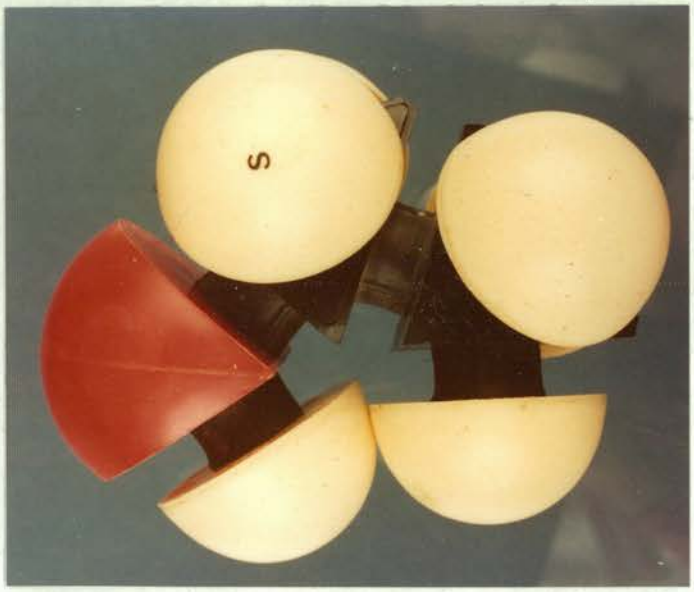
B. Stereochemical representation of the tetrahedral structure.

Fig. 2.1C,D. ETHYL ALCOHOL

C

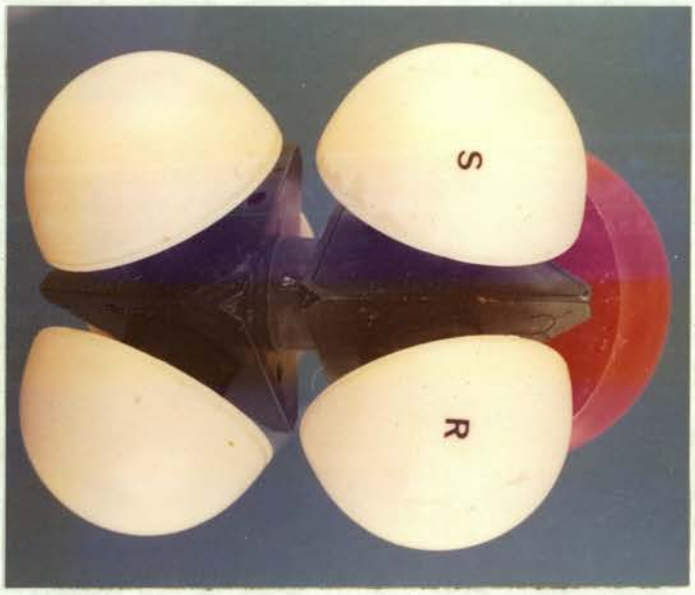


D



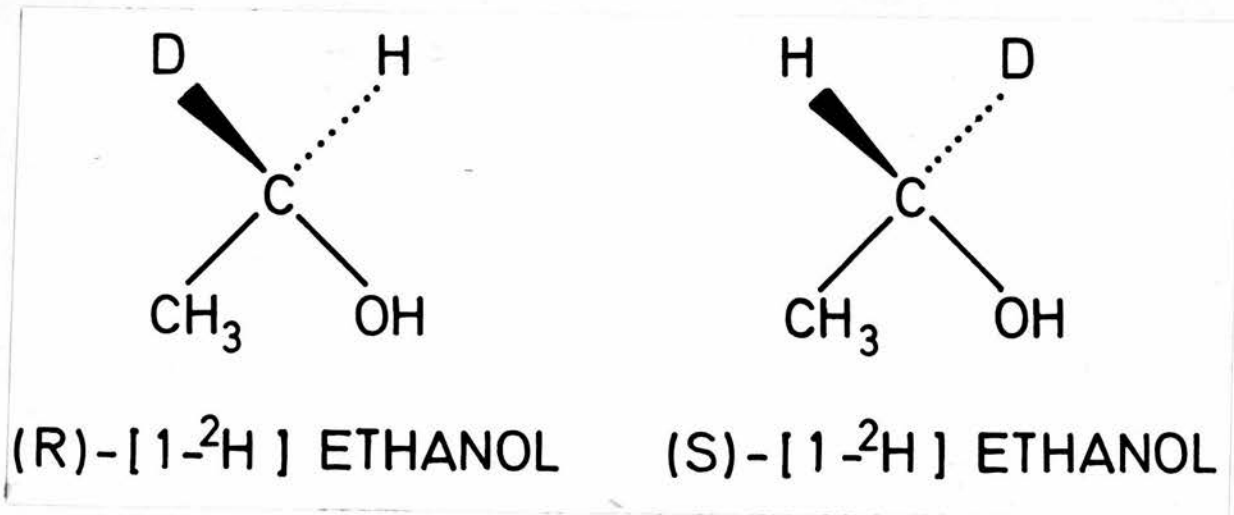
Photographs of molecular models of ethanol C as viewed from in front (i.e. in Fig. 2.1A and B) and D as viewed from behind. The (R) and (S) hydrogen atoms are labelled.

Fig. 2.1E. ETHYL ALCOHOL



Photograph of a molecular model of ethanol as presented in Fig. 2.1A and B but viewed from above. The (R) and (S) hydrogen atoms are labelled.

Fig. 2.2A. THE ENANTIOMORPHS OF [1-^{2}H] ETHANOL



enzymes thus forming NADH (Fig. 2.2.B). This makes position 4 of the dihydronicotinamide ring a pro-chiral centre since if either of the two hydrogen atoms attached to C-4 were replaced with an isotope of hydrogen an asymmetrical centre would be created. Vennesland and her co-workers have provided immutable evidence that yeast ADH reacts in a stereospecific manner in attaching hydrogen to the C-4 position (Vennesland and Westheimer, 1954). Examination of other dehydrogenase enzymes showed them also to be stereospecific and divisible into two groups. One group (group A) transferred hydrogen from substrate to the same face of the ring as yeast ADH but the other group (group B) transferred hydrogen to the epimeric position. The absolute configuration of hydrogen attached in terms of A and B-type dehydrogenases was determined by Cornforth et al. (1962, 1966). In type-A dehydrogenase activity hydrogen is transferred to the A position as shown in Fig. 2.2.B projecting towards the reader. This is the 4(R) position using the notation of Cahn (1964). Conversely with B-type enzymes hydrogen is attached in the B or (S) position. Examples of type-A dehydrogenases include lactate dehydrogenase, malate dehydrogenase, isocitric dehydrogenase and the malic enzyme. The B-group of dehydrogenases includes glucose-6-phosphate dehydrogenase, glucose dehydrogenase, triose phosphate dehydrogenase and squalene synthetase.

The stereospecificity of the ADH reaction is summarised in Fig. 2.3. This shows the oxidation of 1-(R)-D-ethanol. Deuterium is selectively removed from the (R) position of ethanol and transferred to the A or (R)

Fig. 2.2B. THE DIHYDRONICOTINAMIDE RING OF N.A.D.H.

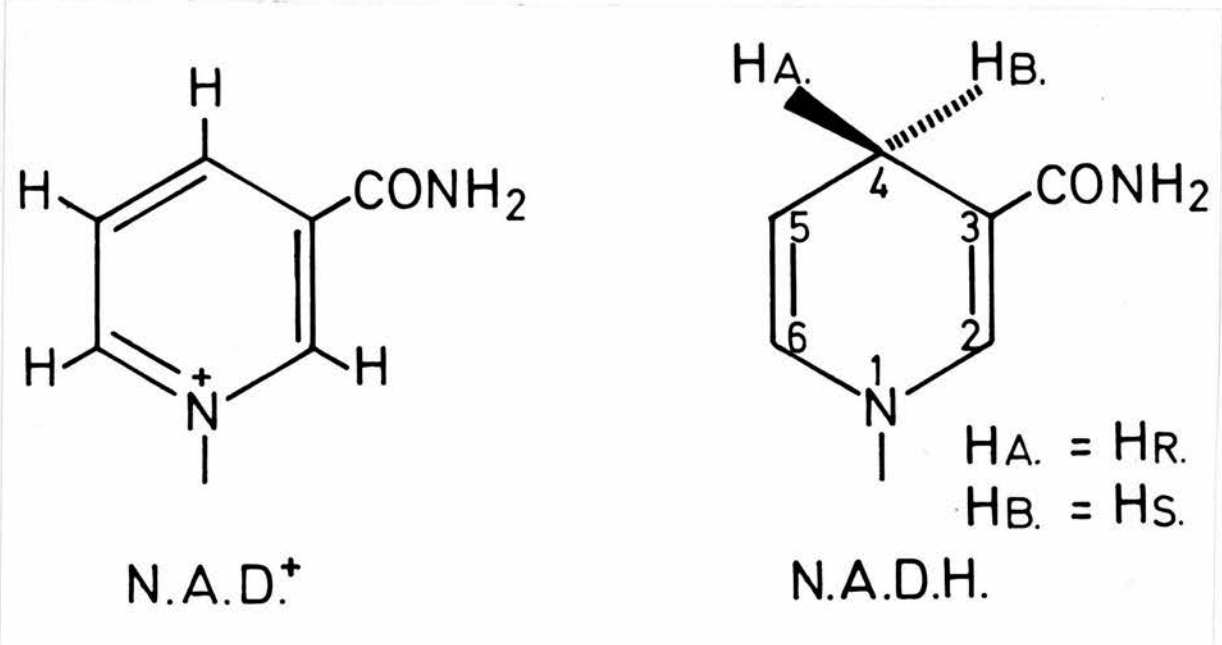
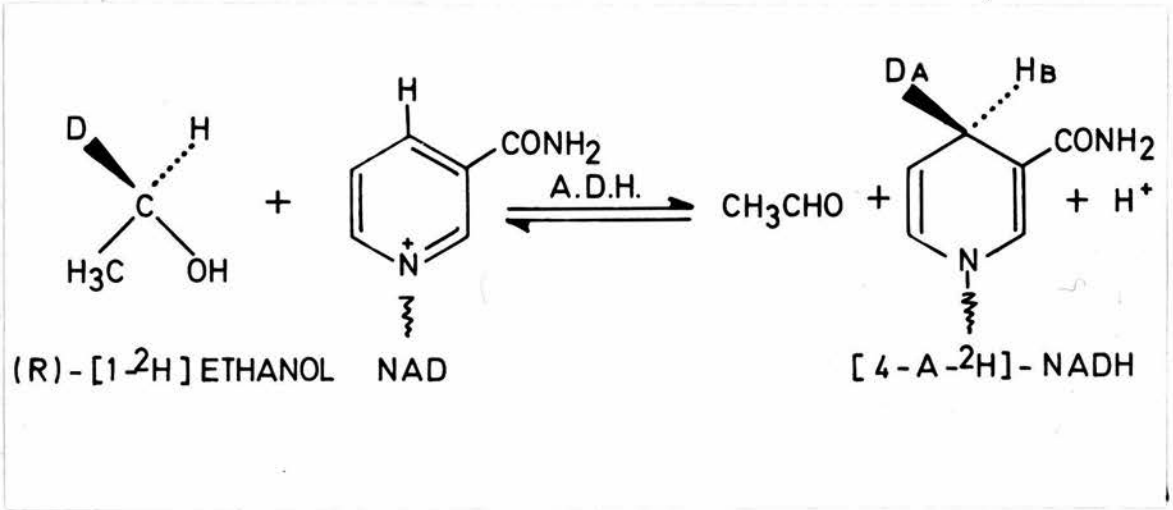


Fig. 2.3. STEREOSPECIFICITY OF HYDROGEN TRANSFER WITH ALCOHOL DEHYDROGENASE



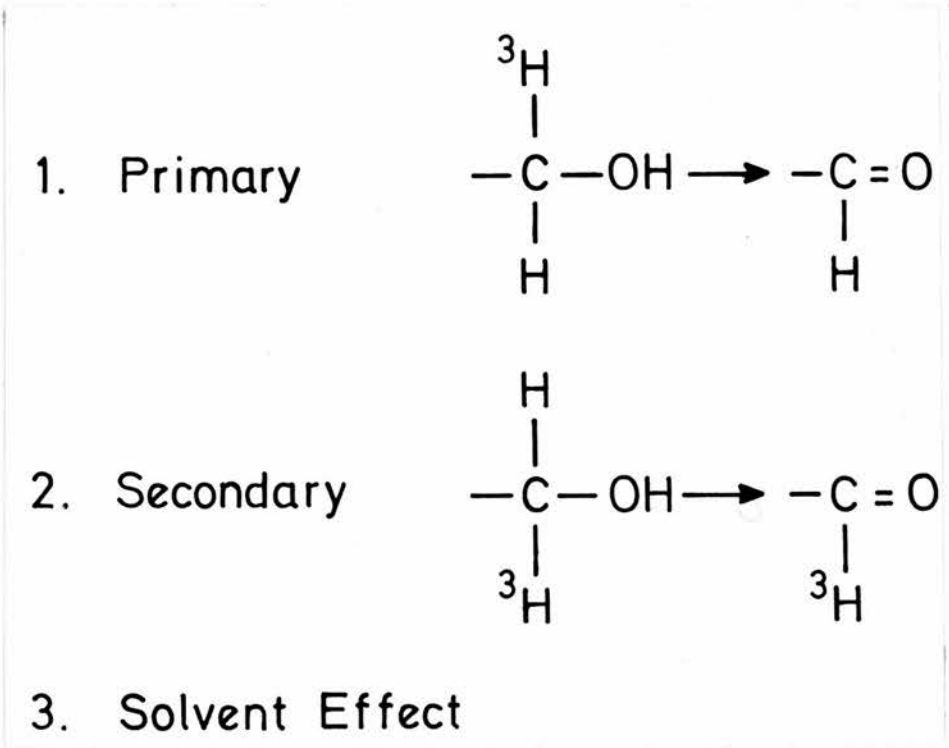
position of NAD forming [4-A-^{2}H]-NADH and acetaldehyde. The same deuterium atom is shuttled between coenzyme and substrate in the reverse reaction.

2. KINETIC ISOTOPE EFFECTS

Introduction

Deuterium, the stable isotope of protium discovered by Urey and co-workers in 1932 constitutes one part in four thousand of hydrogen. This isotope (containing one proton and one neutron in its nucleus) and the radioactive isotope, tritium (containing one proton and two neutrons) have been used extensively as tracers in biological and chemical studies. Generally such isotopes are considered as forms of an element differing only in the number of neutrons in the nucleus and not in ordinary chemical properties. This holds true for qualitative chemical behaviour since the electronic structure of different isotopes of the same element is identical. However two molecular species differing only isotopically may show perceptible differences in the rate constants of chemical reactions. The three types of kinetic isotopic effect are listed in Fig. 2.4. Primary isotope effects result when a covalent bond to the isotope is broken or formed. When an isotopic substitution affects the reaction rate but the formation or rupture of an isotopic bond does not occur this is termed a secondary isotope effect. Therefore in the oxidation of ethanol by ADH the substitution of deuterium in the 1-(R) and 1-(S) positions would result in primary and secondary kinetic isotope effects respectively. Solvent isotope effects may be observed when the reaction takes place in an isotopically substituted solvent phase. For example reactions may occur at a different rate when heavy water (D_2O) is substituted

Fig. 2.4. KINETIC ISOTOPE EFFECTS



for the aqueous phase. This third type of isotope effect cannot be invoked in any of the author's experiments and will not be discussed further. Effects of isotopic substitution on equilibria have been observed but these generally are of much lesser degree than those on rate of reaction.

Primary and Secondary Kinetic Isotopic Effects

Primary isotope kinetic effects result primarily from a reduction of zero point energy in the isotopic species*. A greater activation energy is therefore required to achieve the transition state and the rate of reaction is correspondingly less. For most elements the mass ratios are close to unity (e.g. $^{14}C:^{12}C = 1.17$) and isotopes differ only slightly in chemical behaviour. For hydrogen the mass ratios, Protium: Deuterium: Tritium are 1:2:3 and hence kinetic isotope effects may be very large. These effects may be expressed simply as the ratio of the two rate constants k1/k2. At room temperature for a primary isotope effect the theoretical maximal KH/KD is 7 i.e. an isotope effect of 700% (Wiberg, 1955). The relationship to tritium is as follows:

$KH/KT = (KH/KD)^{1.422}$ (Swain et al. 1955)

By comparison the primary isotopic effect with ^{14}C ranges only as high as 15% and ^{13}C exhibits only onehalf of this (Yankwich et al. 1954).

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*The zero point energy or $E_o = \frac{1}{2}h\nu$ where h = Planck's constant and ν (nu) is the frequency of vibration. The increased mass of isotopes causes a reduction in ν.

Secondary isotope effects are much smaller than primary effects and rarely exceed 30%. They are thought to result if the isotopic bond is strengthened or weakened in achieving the transition state. In addition the C-D bond is slightly shorter than the C-H bond and this may confer different steric and electronic properties.

Hydrogen Isotope Effects in the Oxidation of Ethanol

Isotopically substituted ethanol has been used to elucidate mechanisms of ethanol oxidation. Thus Kaplan in 1954 examined the oxidation of ethanol by bromine obtaining a value for KH/KD of 6.7. He concluded from this that hydrogen was transferred as hydride ion from the methylene carbon to bromine. The enzymatic oxidation of ethanol by ADH *in vitro* has been examined by several groups of workers. Only data obtained with the liver enzyme will be reviewed. Using horse liver enzyme Baker and his coworkers (Baker, 1962; Mahler *et al.* 1962) obtained a KH/KD of 1.6 and Palm *et al.* (1968) a value for KH/KT of 1.3-1.8. On the other hand Gershman and Abeles (1973) with enzyme obtained from mouse and horse liver could not demonstrate any significant kinetic isotope effects. They were unable to explain this discrepancy.

There is good evidence that the rate limiting step of ethanol oxidation by ADH *in vitro* is the dissociation of NADH from the enzyme*. If NADH is removed by a second coupled reaction the oxidation proceeds at a much faster rate (Gupta and Robinson (1966); Raskin and Sokoloff (1968, 1970))

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*For the reaction sequence of ADH see Chapter 1 p. 1.6.

because NADH dissociation is no longer rate limiting. Gershman and Abeles (1973) confirmed this increase in activity with horse and mouse enzyme using lactaldehyde to reoxidise NADH. With the equine enzyme under these conditions a KH/KD of 4.2 was observed. Equivalent isotopic data is not given for the mouse enzyme. This unmasked isotopic effect indicates that when dissociation of NADH is no longer rate limiting the hydrogen transfer step becomes rate determining in vitro and shows a significant primary isotopic kinetic effect. Hydrogen isotope effects observed during ethanol oxidation in vivo will be discussed in chapter 9 (page 9.16- et seq.).

CHAPTER 3: AN ORIGINAL ISOTOPIC METHOD FOR THE QUANTIFICATION OF ETHANOL METABOLISM IN TISSUE

INTRODUCTION AND THEORETICAL BASIS

PRACTICAL CONSIDERATIONS AND EVOLUTION OF THE METHOD

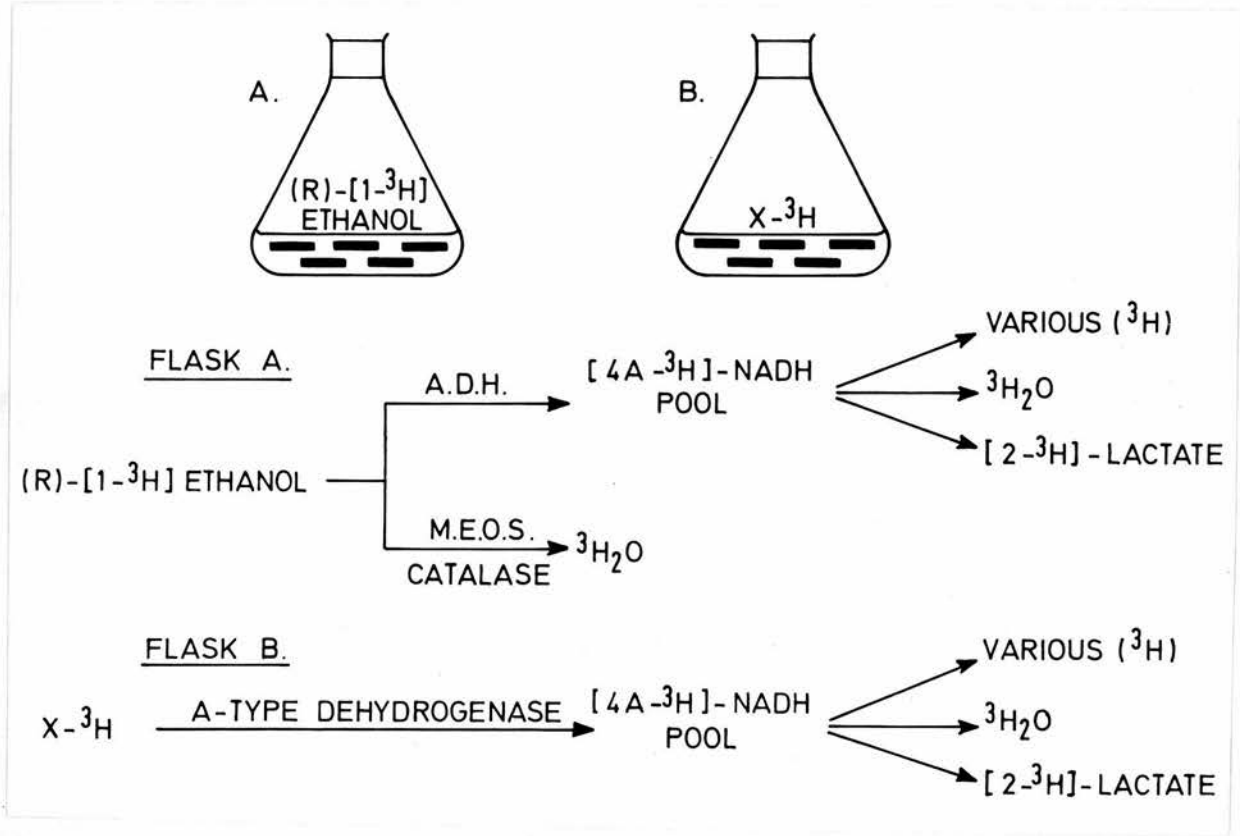
AN ORIGINAL ISOTOPIC METHOD FOR THE QUANTIFICATION OF PATHWAYS OF ETHANOL METABOLISM IN TISSUE

INTRODUCTION AND THEORETICAL BASIS

I have stressed in Chapter 1 the difficulty of quantifying the contribution of the three major oxidative pathways to total ethanol metabolism. In particular problems associated with the use of enzyme inhibitors and subcellular particle preparations have been cited. In order to achieve a meaningful quantification of these pathways I have attempted to develop an isotopic tracer method that does not involve the use of enzyme inhibitors or homogenisation procedures. It is a method that could be used with liver slices, liver perfusion and potentially the whole animal. The purpose of this chapter concluding the first part of this thesis is to explain the evolution and theoretical basis of this method. In the conventional isotopic tracer experiment the distribution of radioactivity from a labelled precursor (usually labelled by isotopic substitution of its carbon skeleton) is traced into different products each considered characteristic of a particular pathway thus enabling a quantification of these pathways. In the oxidation of ethanol all three pathways lead to the same product viz., acetaldehyde and clearly this approach cannot be adopted. In the present method the different pathways are traced indirectly by following the fate of tritium transferred from specifically labelled ethanol during its oxidation.

The theoretical basis will be described in more detail with reference to Fig. 3.1. Two flasks, A and B are incubated each containing medium and an equal quantity of

Fig. 3.1. THEORETICAL MODEL OF AN ISOTOPIC METHOD TO QUANTIFY PATHWAYS OF ETHANOL METABOLISM



liver slices. The media contain "cold" substrates such as glucose, ethanol etc. and are identical in all respects except for their radioactive tracer material. In flask A this tracer is ethanol labelled with tritium in the 1-(R) position. In flask B is a compound X-^{3}H that undergoes oxidation via a NAD-linked dehydrogenase enzyme to produce NADH (labelled with tritium). This is termed the hydrogen donor. The concentration of "cold" hydrogen donor will be identical in both flasks. The two flasks are incubated and allowed to oxidise those two substrates, the reaction is stopped and the media are extracted, assayed and counted. In flask A tritium removed from ethanol via ADH will be transferred to the cytoplasmic NADH pool as NAD^{3}H. Turnover within the pool during the experiment will result in the appearance of tritium in various compounds including lactate (via lactate dehydrogenase) and water (mainly via the mitochondrial cytochrome oxidation system). Tritium removed from (R)- [1-^{3}H] ethanol via catalase and/or MEOS appears exclusively in tritiated water. In flask B all tritium removed is transferred to the NADH pool and will be treated exactly as tritium in the NADH pool in flask A, an identical proportion of that entering being transferred to lactate and water. This assumption that the NADH pools in A and B are identical accrues from the fact that both flasks contain equal weights of liver in the same volume of medium of identical composition and are incubated under identical conditions. It will be evident that in flask A if MEOS and catalase did not exist *in vivo* all tritium would be transferred to NADH and an identical proportion

of that tritium transferred from ethanol would appear in lactate and water as in flask B. Similarly it follows that if alcohol was metabolised exclusively via MEOS and/or catalase all tritium would end up in water and none in lactate. With other proportions e.g. 50% via ADH and 50% via MEOS/catalase then intermediate proportions would be obtained. The development of an equation to calculate the actual proportions is given in Chapter 9 p. 9.10 It should be emphasised that this method cannot distinguish between the separate contributions of catalase and MEOS to ethanol metabolism.

PRACTICAL CONSIDERATIONS AND EVOLUTION OF THE METHOD

Three problems in particular had to be overcome before a sufficiently exact method could be developed:

1. The synthesis of the 2 enantiomorphs of [1-^{3}H] ethanol - these were synthesised and purified by methods described in Chapter 4. Their preparation was necessary to determine the stereospecificities of MEOS and catalase (v. infra) and to use them as radioactive tracer materials in incubation experiments.

2. The determination of the stereospecificity of MEOS and catalase - this information was essential for the selection of suitable radioactive tracers and for the analysis of data from incubation experiments. The stereospecificity experiments are described in detail in Chapters 5 and 6. The data demonstrated that these oxidative mechanisms both exhibit the same stereospecificity as ADH.

3. The selection of a suitable hydrogen donor - this compound should satisfy at least three conditions. Firstly it must be metabolised exclusively by an NAD-linked dehydrogenase enzyme located in the cytoplasm. Secondly it must show A-type stereospecificity as does ADH and lactate dehydrogenase (Loewus et al. 1953) and therefore donate hydrogen (and tritium) to the [4A-H]-NADH pool. Thirdly it should be oxidised only in the liver - thus enabling it to be employed ultimately in the whole animal including man. (S)-[1-^{3}H] ethanol seemed a very promising material to use. Oxidation of this in the cell results in the production of [1-^{3}H] acetaldehyde which would serve as the isotopic tracer. Aldehyde dehydrogenase was considered to be located in the cytoplasm therefore fulfilling the first condition. It shows A-type stereospecificity (Levy and Vennesland 1957) thus satisfying the second condition also. Furthermore from the work of Lundquist et al. (1969) it appeared to be almost exclusively oxidised in the liver. A series of experiments was accordingly undertaken using (S)-[1-^{3}H] ethanol as hydrogen donor. As described in Chapter 7 it proved to be an unsuitable hydrogen donor with which to compare the fate of hydrogen removed from (R)-[1-^{3}H] ethanol. Its unsuitability resulted from a conclusion arrived at from these experiments; that acetaldehyde is not mainly oxidised in the cytoplasm. This interpretation is now supported by evidence obtained by other methods as discussed in Chapter 7.

A review of potential hydrogen donors suggested that [2-^{3}H] sorbitol might serve as a suitable tracer material.

Sorbitol is metabolised solely in the liver by the cytoplasmic enzyme sorbitol dehydrogenase. The stereospecificity of this enzyme was however unknown. I therefore examined this in a series of experiments described in Chapter 8. Fortunately sorbitol dehydrogenase proved to be an A-type enzyme thus fulfilling the second condition. Experiments were then commenced using $[2\text{-}^{3}H]$ sorbitol as hydrogen donor to quantify the pathways of ethanol oxidation to acetaldehyde in rat liver. Details of these experiments are described in Chapter 9.

The method described in this chapter makes two major assumptions. The first is that only one pool of NADH exists in the cytoplasm. Clearly if ADH and sorbitol dehydrogenase transferred tritium into separate cytoplasmic pools of NADH this could invalidate any data. The subject of coenzyme pools is reviewed extensively by Guma et al. (1971) who conclude that only one cytoplasmic NAD/NADH pool does exist although there are probably two such pools in the mitochondria. The second assumption is that kinetic isotope effects do not influence the handling of tritium to a dissimilar degree in different pathways thereby giving a fallacious estimate of their relative contributions.* This possibility is discussed in Chapter 9 page 9.17.

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*The term "isotopic discrimination" is used in the text in later chapters referring to this phenomenon.

THE OXIDATION OF ETHANOL BY MAMMALIAN LIVER

PART II EXPERIMENTAL DATA

CHAPTER 4: THE SYNTHESIS OF THE ENANTIOMORPHS OF [1-^{3}H] ETHANOL

INTRODUCTION

METHOD

MATERIALS

PREPARATION AND PURIFICATION

QUANTITATIVE CONSIDERATIONS

PROOF OF STRUCTURE

STABILITY OF THE ENANTIOMORPHS ON STORAGE

INTRODUCTION

Early in the course of my investigations into ethanol oxidation it became clear that it was necessary to prepare the two tritiated derivatives: (R)-[1-^{3}H] ethanol and (S)-[1-^{3}H] ethanol in acceptably pure form. In producing these compounds advantage was taken of the fact that the enzyme alcohol dehydrogenase shows strict stereospecificity in removing the (R) hydrogen during oxidation of ethanol and in adding hydrogen to the same position during the reverse reductive reaction.

(R)-[1-^{3}H] ethanol was prepared by reducing acetaldehyde using alcohol dehydrogenase and NADH labelled with tritium. This NAD^{3}H was produced by a coupled reaction using lactate dehydrogenase and sodium (R,S)-[2-^{3}H] lactate (Fig. 4.1.A).

(S)-[1-^{3}H] ethanol was prepared in two stages. In the first sodium (R,S)-[2-^{3}H] lactate was oxidatively decarboxylated to [1-^{3}H] acetaldehyde and this was then reduced enzymatically using a similar coupled reaction providing NADH from "cold" sodium lactate (Fig. 4.1B).

METHOD

MATERIALS

Acetaldehyde from Eastman Kodak was stored frozen in aqueous solution at -20^o. Sodium (R,S)-[2-^{3}H] lactate (NET-025) was purchased from New England Nuclear Corporation. The enzyme lactic dehydrogenase - L2625 from beef heart type III and yeast alcohol dehydrogenase - A7011 were purchased from Sigma. Nicotinamide adenine dinucleotide - 15300 CNAA (NAD) was obtained from Boehringer and

Fig. 4.1A. PREPARATION OF (R)-[1-^{3}H] ETHANOL

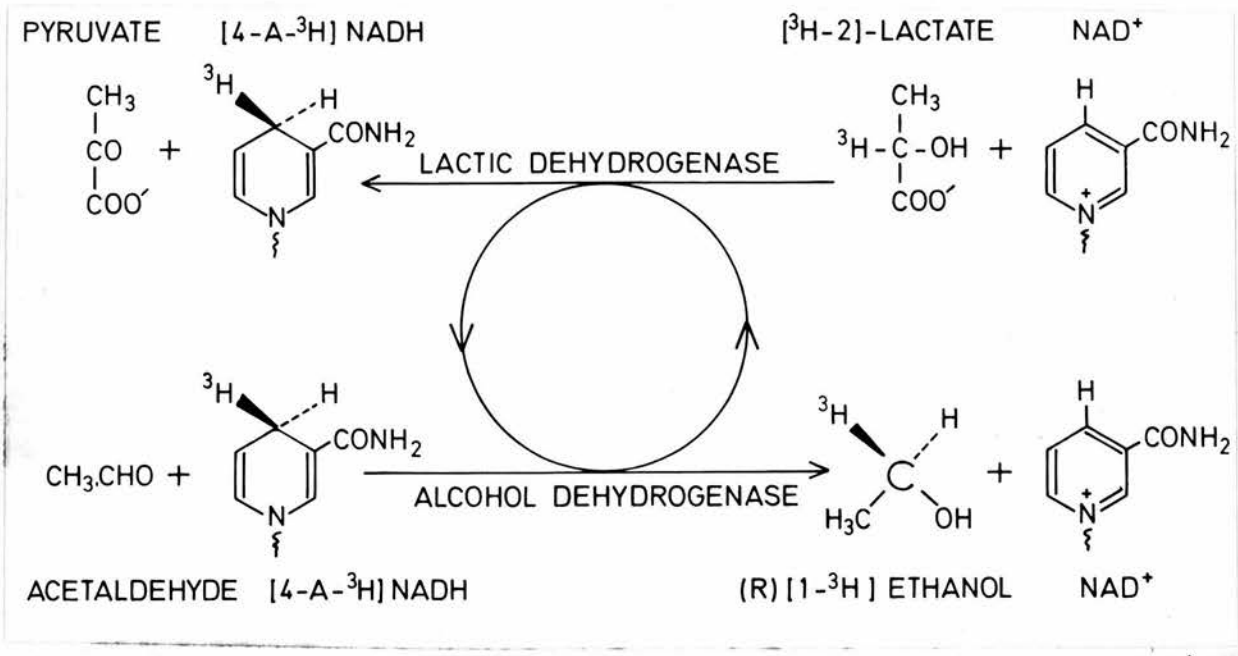
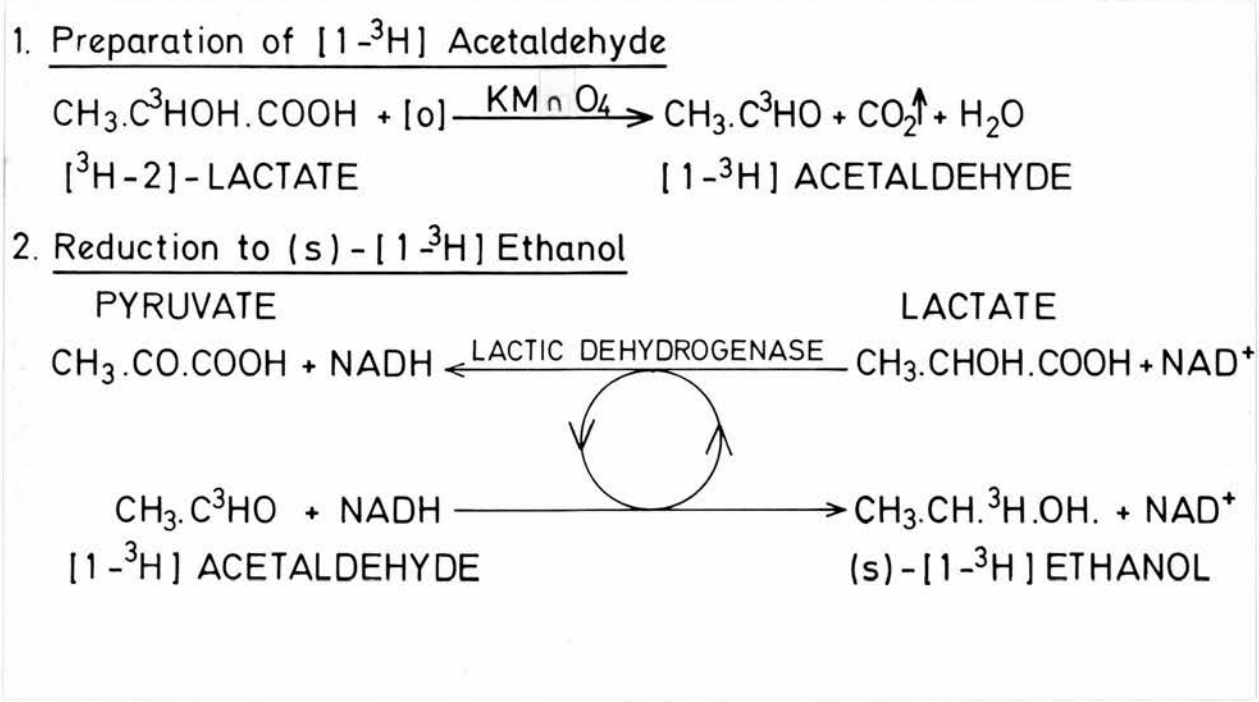


Fig. 4.1B. PREPARATION OF (S)-[1-^{3}H] ETHANOL



the 3-acetyl pyridine analogue of nicotinamide adenine dinucleotide - A5251 (AP-NAD) from Sigma. Dimetol reagent was made by dissolving dimethylcyclohexanedione (Eastman Kodak) 4 mg per ml in acetate buffer, pH 4.2, 0.1M. Counting was performed on a Nuclear Chicago 727 scintillation counter. All counts were converted to disintegrations per minute (d.p.m.) by internal standardisation.

PREPARATION AND PURIFICATION

(R) -[1-^{3}H] Ethanol was prepared by the enzymatic reduction of acetaldehyde using sodium (R,S)-[2-^{3}H] lactate with lactic dehydrogenase and alcohol dehydrogenase. In a stoppered flask 1 mg of lactic dehydrogenase, 0.65 mg of alcohol dehydrogenase, 1.25 mg of NAD, 9.65 mg of lithium (R,S)-lactate, 7mCi of sodium (R,S)-[2-^{3}H] lactate, and 32 mg of acetaldehyde were incubated at 37^o in 9.65 ml of phosphate buffer, pH 7.4, 0.1M. After 3 hours the reaction was terminated by the method of Somogyi (1945).

(S)-[1-^{3}H] Ethanol was prepared in two steps. In the first step, 9.6 mg of lithium (R,S)-lactate with 8mCi of sodium (R,S)-[2-^{3}H] lactate was oxidatively decarboxylated to [1-^{3}H] acetaldehyde (Brin and Olson, 1952). This was collected in 5 ml of ice-cold water. In the second step, the aqueous [1-^{3}H] acetaldehyde was added to 10.7 ml phosphate buffer, pH 7.4, 0.2M, containing 160 mg of lithium (R,S)-lactate, 1.25 mg of NAD, 0.65 mg of alcohol dehydrogenase, and 1 mg of lactic dehydrogenase. This was incubated in a stoppered Erlenmeyer flask at 37^o for 3 hours when the reaction was terminated (Somogyi, 1945).

Both ethanols were collected in aqueous solution by distillation. Contaminating acetaldehyde was removed by combination with semicarbazone and the ethanols were redistilled under reduced pressure at 20^{o}.

By a precipitation technique (v. infra) it was shown that less than 0.6% of the d.p.m. in tritium was present in contaminating acetaldehyde. With experiments in which a high proportion of the ethanol undergoes oxidation this degree of contamination would probably have negligible effects. However if only a small fraction of ethanol in the medium is oxidised it could exert a quantitatively significant influence on the d.p.m. counted in acetaldehyde formed and thereby invalidate data. In such circumstances the amount of contamination can be reduced greatly by repeated purifications with semicarbazone as described in Chapter 6. The quantitative effect of these additional d.p.m. in acetaldehyde has been allowed for in all experiments by the use of appropriate controls.

QUANTITATIVE CONSIDERATIONS

In the preparation of (R)-$[1-^{3}H]$ ethanol commencing with 7mCi of sodium (R,S)-$[2-^{3}H]$ lactate 2.42 mg of ~~cold~~ ethanol and 1.32mCi of radioactivity were produced representing 105% and 38% of theoretical yields respectively. The yield of (S)-$[1-^{3}H]$ ethanol starting with 8mCi of sodium (R,S)-$[2-^{3}H]$ lactate was 2.8mg ~~cold~~ ethanol (60% of theoretical) containing 2.92mCi of radioactivity (37% of theoretical).

PROOF OF STRUCTURE

The putative structure was tested by oxidation to acetaldehyde using alcohol dehydrogenase with 3-acetyl pyridine NAD (AP-NAD) as coenzyme in pyrophosphate buffer, pH 9.0, 0.06M. Initial experiments using NAD had resulted in an insignificant oxidation of the ethanol and AP-NAD was substituted because of its more favourable equilibrium. The acetaldehyde was distilled into dimetol reagent and precipitated overnight as dimetoacetaldehyde (Brin and Olson 1952). This was washed with 500 ml of water in a Buchner funnel and dried at 60^{o} under reduced pressure. The weighed precipitate was dissolved in Aquasol and counted. The specific activity of the acetaldehyde (Table 4.1) approximated closely to that of the parent ethanol in the case of (S) $[1-^{3}H]$ ethanol. In contrast, when (R)-$[1-^{3}H]$ ethanol was the substrate, only 1.85% of the tritium was retained in the acetaldehyde.

Some or all of this tritium may be explicable on the basis of contaminating tritiated acetaldehyde. However the possibility exists that at least some of this radioactivity represents tritium in the (S) position and suggests that alcohol dehydrogenase does not show absolute stereospecificity. Each of the enantiomers was oxidized to acetic acid by chromic acid. The acetic acid from each contained less than 0.09% of the radioactivity of the corresponding ethanol, indicating that this ectopic tritium is not in the 2 position.

TABLE 4.1

OXIDATION OF ENANTIOMERS OF [1-^{3}H] ETHANOL BY YEAST ALCOHOL DEHYDROGENASE USING 3-ACETYL PYRIDINE N.A.D.

	Ethanol Specific Activity	Acetaldehyde Specific Activity	Ratio of Specific Acetaldehyde to Specific Activity Ethanol[c]
(R)-[1-^{3}H] ethanol	1.4	0.026	0.019
(S)-[1-^{3}H] ethanol	1.79	1.81	1.01

Results of the oxidation of the tritiated ethanols are expressed as the specific activity dpm X 10^{-6} per mmole of the alcohol incubated with alcohol dehydrogenase and the specific activity of the acetaldehyde produced.

STABILITY OF THE ENANTIOMORPHS ON STORAGE

Aqueous solutions of ethanol are known to undergo spontaneous oxidation to acetaldehyde. Furthermore acetaldehyde has been recognised as a major product of the radiolysis of ethyl alcohol (McDonnell and Newton, 1954). By addition to the two enantiomorphs of an excess of dimetol reagent and a known amount of "cold" acetaldehyde it is possible to estimate the percentage of counts in acetaldehyde counted as its precipitated dimedone derivative. It was thus possible to demonstrate a breakdown to radioactive acetaldehyde of the two enantiomorphs during storage at -20^{o}C. This resulted in proportions sufficient to interfere with the results of isotopic tracer experiments. In view of this phenomenon periodic checks were kept on the degree of contamination and when necessary repurification was performed as described above.

CHAPTER 5: STEREOSPECIFICITY OF THE OXIDATION OF ETHANOL BY CATALASE

INTRODUCTION

MATERIALS

METHOD

RESULTS

DISCUSSION

INTRODUCTION

Keilin and Hartree (1955) showed that catalase was capable of oxidizing a wide variety of compounds including ethanol in the presence of a hydrogen peroxide generating system. This peroxidatic oxidation is discussed in detail in Chapter 1 (page1.8 to 1.11). The possibility that catalase might be involved in ethanol oxidation *in vivo* necessitated the following experiments to determine the stereospecificity of this peroxidatic oxidation of ethanol. The method was modified after that of Keilin and Hartree (1955).

MATERIALS

Glucose oxidase - NG6125 Type II and catalase - C100 from beef liver, twice recrystallized were purchased from Sigma. Dimetol reagent was made by dissolving dimethyl-cyclohexanedione (Eastman Kodak) 4 mg per ml in acetate buffer, pH 4.2, 0.1M. Counting was performed on a nuclear Chicago 727 scintillation counter. Aquasol (New England Nuclear Corporation) was used as scintillant. All counts were converted to disintegrations per minute by internal standardisation.

METHOD

In a stoppered 50 ml pear-shaped flask 8 mg of ethanol with 0.1 uCi of tritium, 1 mg of catalase, 0.2 mg of glucose oxidase, and 60 mg of glucose were incubated at 25^{o} in 12 ml of potassium phosphate buffer, pH 5.9, 0.2M, for 15 hours. The flask was connected to a distillation apparatus and 5 ml were distilled into 8 ml of ice-cold dimetol reagent and the weighed precipitate was counted as described on p. 4.4. Identity of the dimetoacetaldehyde

was confirmed by melting point determination (139°). Appropriate control experiments without catalase and with added carrier acetaldehyde were performed to allow correction for contaminating [1-^{3}H] acetaldehyde. A 0.1 ml aliquot was removed from the flask at termination, deproteinated, and assayed for ethanol (Bonnichsen, and Theorell, 1951), to determine the percentage of oxidation. In a separate experiment it was shown that the ethanol disappearance could be entirely accounted for by the accumulation of acetaldehyde assayed by the method of Gupta and Robinson (1966).

RESULTS

The results of peroxidatic oxidation are shown in Table 5.1. The specific activity of the resultant acetaldehyde on oxidation of the (S)-[1-^{3}H] ethanol approximated closely to that of the ethanol. With the (R)-[1-^{3}H] ethanol it was less than 3% of that of the ethanol. These results parallel those obtained with oxidation by alcohol dehydrogenase (Table 4.1). To eliminate the possibility of oxidation via contaminating alcohol dehydrogenase, the following experiment was performed. Catalase was added to a pyrophosphate buffer, pH 9.4, 0.075M, containing ethanol, NAD, and semicarbazone and was incubated for 30 min at 37°. No change in optical density at 340nm was observed after the addition of catalase. In a control flask to which alcohol dehydrogenase was added, a change in optical density of 0.67 was observed.

DISCUSSION

These peroxidatic oxidation experiments provide the first incontrovertible information on the stereospecificity

TABLE 5.1

PEROXIDATIC OXIDATION OF ETHANOL

Experiment	^3H Position	Per cent Ethanol Oxidized	Ethanol Specific Activity	Acetaldehyde Specific Activity	Ratio of Specific Activity Acetaldehyde to Specific Activity Ethanol
1	R	95	1.37	0.028	0.020
	S	95	1.81	1.69	0.93
2	R	75	1.20	0.032	0.027
	S	84	1.31	1.32	1.01
3	R	77	1.20	0.030	0.025
	S	75	1.31	1.28	0.97

Specific activities of the ethanols and the acetaldehydes formed from them are expressed as dpm X 10^{-6} per mmole. Corrections have been made for the contributions of contaminating tritiated acetaldehyde (not more than 0.6% of the added dpm) in the [1-^3H] ethanol preparations.

of catalase. Table 5.1 indicates that the pro-R-hydrogen atom is selectively removed during peroxidatic oxidation. Vennesland and her co-workers (see Vennesland and Westheimer, 1954), showed that alcohol dehydrogenase has an identical stereospecificity. This strict stereospecificity of catalase is remarkable in view of its broad spectrum for substrates including formaldehyde and nitrite (Keilen, 1955). The results are clearly not an artifact of alcohol dehydrogenase contamination as shown by the experiment cited with NAD at pH 9.4. Furthermore, it is inconceivable that alcohol dehydrogenase could achieve almost complete oxidation of ethanol at pH 5.9 in the absence of a trapping agent for acetaldehyde. This stereospecificity of catalase may explain at least in part the high degree of orientation of the ethanol molecule with catalase that Sizer (1944) predicted was necessary on thermodynamic grounds.

Following the completion of the above work Gang et al. (1973) published a preliminary communication on the stereospecificity of ethanol oxidation. These workers also found evidence for selective removal of the (R)-hydrogen by catalase. However their methods may be criticised on several grounds as discussed in Chapter 6 page 6.10.

CHAPTER 6: STEREOSPECIFICITY OF THE MICROSOMAL ETHANOL OXIDISING SYSTEM

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION

INTRODUCTION

In order to follow the scheme outlined in Chapter 3 for the quantification of pathways of ethanol metabolism *in vivo* it was necessary to determine the stereospecificity of ethanol oxidation by the microsomal ethanol oxidising system (MEOS). Unlike catalase or alcohol dehydrogenase this cannot be isolated in pure form, consisting as it does of a complex membrane-bound multi-enzyme and cofactor system. Instead it has been examined in the form of various microsomal suspensions prepared by different methods and of varying degrees of purity, contaminants of particular importance being catalase and alcohol dehydrogenase.

I have examined the preparations of Lieber and DeCarli (1970) and Isselbacher and Carter (1970). Clearly contamination by catalase or alcohol dehydrogenase in microsomal suspensions could lead to the false impression of removal of the (R) hydrogen atom by MEOS. In order to avoid this problem I prepared a "washed" suspension involving further purification steps. I have also examined ethanol oxidation in the catalase-free, P450 rich preparation of Mezey *et al.* (1973).

In experiments *in vitro* with catalase or alcohol dehydrogenase it is possible to obtain an almost quantitative conversion of alcohol to acetaldehyde by choosing appropriate conditions. In microsomal suspensions the activity is much lower (with the exception of the monkey - v. infra) and it was necessary to modify the method. It was not possible merely to count acetaldehyde formed on oxidation

of ethanol as an insoluble derivative because of its very low yield. Instead [2-^{14}C] ethanol was added to the incubation medium and after stopping the reaction "cold" acetaldehyde was added as carrier and the insoluble acetaldehyde derivative was subsequently counted for tritium and ^{14}C. By changes in ^{3}H/^{14}C ratios it was possible to draw conclusions concerning the stereospecifidty of hydrogen removal. Also in view of the small fraction of alcohol in the medium that was oxidised it was necessary to use the enantiomorphs of [1-^{3}H] ethanol in a very high state of purity i.e. with minimal contamination by [1-^{3}H] acetaldehyde.

MATERIALS AND METHODS

Radio-chemicals

(R)-[1-^{3}H]ethanol and (S)-[1-^{3}H]ethanol were prepared as detailed in Chapter 3. They were purified by repeated distillation with semicarbazone until less than 0.01% of the dpm was present in [1-^{3}H] acetaldehyde as a contaminant. [2-^{14}C]ethanol was purchased from New England Nuclear Corporation, Boston, Massachusetts. This was found on testing to have more than 2% of the dpm present as [2-^{14}C]acetaldehyde. It was accordingly purified by the same distillation method to a similar degree of purity.

Microsomal Preparation

Liver of the rat and monkey was used for microsomal preparations. Female Sprague-Dawley rats (Charles River, Wilmington, Massachusetts) were used weighing between 200 and 300 grammes. Livers were obtained from three monkeys that were being sacrificed for unrelated studies. The first monkey, a male, had been fasted 72 hours and his

liver was removed under phencyclidine anaesthesia. The other two monkeys both females, had been fasted overnight, and were under barbiturate anaesthesia. The first monkey had undergone a 48 hour period of hypothermia one year previously. The second was sacrificed following accidental haemorrhage. The third monkey had undergone a previous oophorectomy. It is assumed that these circumstances do not alter the stereospecificity of ethanol oxidation but might change the quantity of ethanol utilised. The livers were collected in cold isotonic saline and homogenised within 40 minutes of removal.

Microsomes were prepared by the published methods of Isselbacher and Carter (1970) and Lieber and DeCarli (1970). The important stages of these methods are compared in Table 6.1. Microsomes were also prepared by an original method referred to as a "washed" preparation (Table 6.1 extreme right hand column). This follows the method of Isselbacher in the initial stages but incubation is carried out in potassium phosphate buffer in the presence of air and not oxygen. In addition there are two further centrifugations at 105,000g following resuspension of the microsomal pellet to its original volume. These centrifugations are undertaken to purify the microsomes from contaminants such as cytoplasmic alcohol dehydrogenase or catalase originating from peroxisomes. In some experiments incubation was carried out with microsomes harvested after the first centrifugation at 105,000g. These microsomal suspensions are referred to in Table 6.2 as "crude".

TABLE 6.1

PREPARATION OF MICROSOMES BY THREE DIFFERENT METHODS

Method	Isselbacher et al.	Lieber et al.	"Washed"
Homogenisation	20% in 0.25M Sucrose	20% in 1.15% KCl	20% in 0.25M Sucrose
Initial Centrifugation	20,200xg 20 minutes	9,000xg 30 minutes	20,200xg 20 minutes
Subsequent Centrifugation	105,000xg 1 hour	100,000xg 1 hour	105,000xg 1 hour x 3
Incubation Medium	80 mM Na Phos. 7.4 in O_2	80 mM K. Phos. ph 7.4 in Air	80 mM K. Phos. pH 7.4 in Air
TPNH Source	TPNH	Na Isocitrate/ISO cit. D.H.	TPNH

Catalase-free MEOS

The procedure was modified after that of Mezey et al. (1973). After isolation of the microsomal pellet and its solubilisation, the ammoniumsulphate precipitate was dialysed against two changes of buffer for 8 to 12 hours. The dialysed protein was passed through a Sephadex-G-25 column and was then placed on a DEAE cellulose column and eluted with KCl. As observed by Thurman and Scholz (1972), Teschke et al. (1972), and Mezey et al. (1973), catalase activity appeared at the beginning of elution and a cytochrome P-450 peak followed which was devoid of catalase activity. The tube fractions containing the P-450 peak were combined and ammoniumsulphate was added to 50% saturation. The resulting precipitate was collected and dissolved in potassium phosphate buffer. Catalase activity was determined by the method of Lück (1965); Cytochrome P-450 by the method of Omura and Sato (1964).

Incubation Procedure

Except for the catalase-free preparation (v. infra), incubation of the microsomal suspension was in 250 ml Erlenmeyer flasks. The incubation conditions for the preparations of Isselbacher and of Lieber were followed exactly. In other preparations incubation was with a phosphate buffer (80mM) at pH 7.4 with 0.3mM NADPH and 5mM magnesium chloride with a final protein concentration of 3 mg/ml.

Except for the preparation of Isselbacher microsomal suspensions were exposed to air for 10 minutes at 37°. Labelled ethanol was then added and the flasks were

stoppered for incubation. The preparation of Isselbacher was stoppered and gassed with oxygen at 37° through sealed glass inlets prior to injection of labelled alcohol. In all experiments 0.4 - 1.2 uC of 2-^{14}C ethanol was added to each flask and 1 - 3 uC of (R)-[1-^{3}H]ethanol added to one flask and 1 - 3 uC of (S)-[1-^{3}H]ethanol to the other flask. The ethanol was added in a quantity to give a final concentration in the 40 ml volume of 2.3 mg/ml. The flasks were incubated for 20 minutes. In two control flasks boiled microsomal suspension or 0.15M KCl were substituted for active microsomes. In incubating the catalase-free MLOS preparation the total volume of the reaction mixture was 5 ml, the protein concentration was 13 mg/ml, and 50 ml Erlenmeyer flasks were used.

Isolation of Acetaldehyde

Incubation was terminated by the addition of 1 ml of 0.3N $Ba(OH)_2$ and 1 ml of 5% $ZnSO_4$ to the flasks and then 4.8 mg of unlabelled acetaldehyde in 0.3 ml of water was injected. The content of each Erlenmeyer flask was cooled and poured into a screw-cap plastic centrifuge bottle which was sealed and spun at 5,000g for 30 minutes in a refrigerated centrifuge. The supernatant was transferred to a pear-shaped flask and distilled. The first 5 ml of distillate was collected in 8 ml of ice-cold dimetol reagent. The precipitate of dimetoacetaldehyde was collected, washed, dried, and weighed (Chapter 4, p. 4.4).

Radioactivity Assays and Calculations

The weighed precipitate of dimetoacetaldehyde was dissolved in Aquasol^{R} (New England Nuclear Corporation,

Boston, Mass.) and counted on a Nuclear Chicago 727 Scintillation Counter. The total dpm in ^{3}H and ^{14}C were determined by internal standardisation. The dpm incorporated into acetaldehyde was calculated from the weight of dimetoacetaldehyde collected and the 33.4 mg of dimetoacetaldehyde to be expected from 4.8 mg of acetaldehyde, if there were no losses during the process of isolation. Between 16 and 19 mg of dimetoacetaldehyde were collected. Samples of the media employed were counted to obtain the total dpm of ^{14}C and ^{3}H added to the flask.

^{14}C and ^{3}H were present in the dimetoacetaldehyde isolated in the control incubations. This radio-activity was the same whether boiled microsomes or 0.15M KCl were substituted for active microsomes. It was the same even if the reaction was terminated prior to the addition of isotope and incubation. It appears therefore to result from contamination of the labelled ethanol by traces of radioactive acetaldehyde. By using radio-chemicals of high purity (see page 6.2) this contamination was less than 10% of the total radioactivity counted in dimetoacetaldehyde derived from the active microsomal preparations. To allow for this contamination the dpm in acetaldehyde in the control flasks were subtracted from the corresponding dpm in the acetaldehydes formed by MEOS. By incubating catalase-free MEOS at a protein concentration of 13 mg/ml, contamination by labelled acetaldehyde was also a minor quantity compared to the amount of labelled acetaldehyde formed, despite the lower MEOS specific activities in these preparations.

RESULTS

In presenting the data the $^{3}H:^{14}C$ ratio of the acetaldehyde formed is divided by the same ratio of the added radioactive ethanol. If the ^{3}H-labelled hydrogen was retained in the formation of acetaldehyde, assuming no isotopic effects the $^{3}H:^{14}C$ ratio in the acetaldehyde would be the same as in the ethanol from which it was formed, and hence this value should equal unity. If the ^{3}H-labelled hydrogen was completely lost in the formation of the acetaldehyde, this calculated ratio should be zero.

Nanomoles of acetaldehyde formed per minute of incubation per mg of microsomal protein has been calculated from the specific activity of the $[2\text{-}^{14}C]$ethanol and the quantity of ^{14}C incorporated into the acetaldehyde. The quantity formed in the flask containing (R)-$[1\text{-}^{3}H]$ethanol was within 10% of that in the flask with (S)-$[1\text{-}^{3}H]$ethanol in each experiment, and the average of these two values is recorded.

The results of the experiments are recorded in Table 6.2. In the rat two experiments were performed under the conditions of Lieber and DeCarli, and two under those of Isselbacher and Carter. In these experiments there was 14% or less retention of the (R)-hydrogen, while the retention of the (S)-hydrogen ranged from 64% to 100%.

Similar results were obtained in both species with "crude" and "washed" preparations. Thus little or no tritium was present in acetaldehyde formed from (R)-$[1\text{-}^{3}H]$ethanol, while 80% or more of the tritium was retained with oxidation of ethanol labelled in the 1-(S) position. There is an indication that in the monkey the retention of the (S)-hydrogen

TABLE 6.2

OXIDATION OF (R) AND (S) 1-TRITIATED ETHANOLS WITH $[2\text{-}^{14}C]$ ETHANOL BY MICROSOMAL PREPARATIONS

Species	Microsomal Preparation	Expt. Number	^3H: ^{14}C of Acetaldehyde / ^3H: ^{14}C of Ethanol		C_2H_5OH oxidized nmoles/min/mg protein
			(R)-$[1\text{-}^3H]$	(S)-$[1\text{-}^3H]$	
Rat	Isselbacher	1	0.00	0.79	2.1
		2	0.10	0.64	1.4
	Lieber	1	0.14	0.82	3.5
		2	0.13	1.00	2.8
	"Washed"	1	0.03	0.87	0.7
		2	0.16	0.81	1.4
	Catalase-free	1		0.81	0.6
		2	0.07		0.4
Monkey	"Crude"	1	0.07	0.98	453
		2	0.05	1.03	258
		3	0.03	1.13	151
	"Washed"	1	0.02	0.95	73.8
		2	0.02	0.88	8.2
		3	0.03	0.91	12.5
	Catalase-free	1	0.02	0.80	1.5

and the loss of the (R)-hydrogen were more complete. The quantity of acetaldehyde formed per minute per mg of protein was greater with the monkey preparations. Little change was noted in acetaldehyde formation as a result of the repeated washings of the rat microsomal preparation before incubation. However a significant reduction is seen in the monkey as a result of this procedure. The specific activity in terms of nanomoles of acetaldehyde formed per mg of protein was least with the catalase-free preparation. Data obtained with this preparation is also compatible with removal of the (R)-hydrogen.

DISCUSSION

There are differences between the preparative methods of Leiber and DeCarli and Isselbacher and Carter (Table 6.1). In particular Isselbacher incubated microsomes in the presence of oxygen which may affect catalase peroxidative activity. On the other hand Leiber adds an impure form of isocitric dehydrogenase which is a possible cause of enzyme contamination. Therefore because of these differences in method and the disparate results obtained by these two investigators it was decided to examine the stereospecificity of both preparations.

In all the circumstances the results (Table 6.2) indicate a retention of the (S)-hydrogen and a loss of the (R)-hydrogen during the oxidation of ethanol to acetaldehyde by the microsomal system. Using the rat preparations there is a suggestion that there is a small retention of the (R)-hydrogen and incomplete retention of the (S)-hydrogen, and this may indicate oxidation by more than one reaction.

With the monkey preparations the calculated ratio with the (R)-hydrogen appears somewhat closer to 0, and with the (S)-hydrogen closer to 1.0. Secondary isotopic effects could be responsible for the failure to obtain complete retention of the (S)-hydrogen. In other words the presence of tritium in the (S) position might interfere with the removal of the (R) hydrogen atom from the ethanol molecule. The approach to the theoretical calculated ratio of 1.0 using crude monkey preparations may be due to the higher percentage of conversion of ethanol in these incubations, so that a kinetic isotopic effect would be obscured. In addition the following are possible contributory mechanisms to explain these data:

1. The presence of ectopic tritium in the two enantiomorphs could introduce an error. However evidence quoted in Chapter 4 indicates that such radioactivity constitutes at the most 2% of the total tritium present.
2. Statistical inaccuracy in estimating $^{3}H:^{14}C$ ratios could explain some discrepancy from theoretical values.
3. If some of the acetaldehyde formed was oxidised by a system exhibiting a kinetic isotope effect this could modify the $^{3}H:^{14}C$ ratio of the precipitated acetaldehyde.

Gang et al. (1973) reported a single experiment showing that racemically labelled [1-^{3}H]ethanol on incubation with rat MEOS yields acetaldehyde with considerably less than half of the specific activity of the [1-^{3}H]ethanol. This would suggest a significant degree of Secondary isotopic discrimination for the (S)-hydrogen. In addition, the R-hydrogen from (R)-[1-^{3}H] ethanol was apparently removed

resulting in no discernible counts in acetaldehyde. This communication is open to criticism on several grounds including the following: 1. The putative structure of (R)-[1-^{3}H]ethanol was not established nor was freedom from contamination by [1-^{3}H] acetaldehyde.

2. The racemate was not examined for contamination by [1-^{3}H] acetaldehyde.

3. Dimetoacetaldehyde was made by adding the entire reaction mixture to Dimetol reagent and the identity of the precipitate was not adequately confirmed.

4. Only one isomer of [1-^{3}H] ethanol was used so that a direct demonstration of retention of the (S) tritium was not possible.

5. The quantity of ethanol oxidised was not reported.

Nevertheless the data, subject to the above reservations do support the removal of the (R)-hydrogen atom by MEOS.

Acetaldehyde formation in our preparations was less than the 14.7 nanomoles of protein/min/mg reported by Lieber and DeCarli (1972) for a 10-minute incubation period. In expressing our results we have assumed linear formation of acetaldehyde over the 20-minute period of incubation (Lieber and DeCarli 1970). The greater rate of formation of acetaldehyde in the monkey could suggest that in the primate MEOS is more active. However, Mannering et al. (1969) reported that monkey liver has a larger quantity of catalase than the rat, but it is less active peroxidatively. MEOS prepared from human liver obtained by biopsy is reported to have somewhat less ethanol oxidizing activity than MEOS prepared from rat liver (Lieber and DeCarli 1970).

The amount of ethanol oxidized in the incubation with MEOS from monkey liver exceeds by many fold the amount of NADPH added. Vatsis and Schulman (1973) made a similar observation and postulated the existence of a constituent in microsomes that regenerates NADPH during ethanol oxidation.

The stereospecificity of MEOS, the removal of the (R)-hydrogen in acetaldehyde formation is the same as that of alcohol dehydrogenase (see Chapter 2) and catalase (see Chapter 4). If it had been different it would have indicated that MEOS was a unique system for oxidising ethanol. That it has the same stereospecificity as catalase is in accord with catalase participating in the MEOS system (Chapter 1 p.1.19). However we have been able to observe acetaldehyde formation with the same stereospecificity in a MEOS preparation in which catalase was not detected by assay (Luck, 1965) albeit with the formation of less acetaldehyde than in the catalase-containing preparations. These three oxidative pathways would therefore appear to orientate the ethanol molecule so that the same hydrogen atom is removed during oxidation by apparently different enzymatic mechanisms. Furthermore in the light of the report (Hrycay and O'Brien 1971) that P450 possesses peroxidative activity it is perhaps not surprising that catalase and MEOS exhibit the same stereospeceficity.

CHAPTER 7: OXIDATION OF THE ENANTIOMORPHS OF [1-^{3}H] ETHANOL BY RAT LIVER IN VITRO

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION

INTRODUCTION

The two enantiomorphs of [1-^{3}H] ethanol had now been prepared and the stereospecificity of hydrogen removal by catalase and MEOS determined. In accordance with the scheme set out in Chapter three the next step was to examine the fate of tritium removed from these two derivatives during oxidation in liver. (S)-[1-^{3}H] Ethanol would be oxidised in the cell to [1-^{3}H]acetaldehyde. It was assumed that during the oxidation of [1-^{3}H] acetaldehyde all tritium would be transferred to the cytoplasmic [4A-H]-NADH pool as discussed on page 3.4. (S)-[1-^{3}H] Ethanol would thus represent X-^{3}H in Fig. 3.1. The principle therefore was to utilise (S)-[1-^{3}H] ethanol as a donor to the [4A-H]NADH pool with which to compare the fate of tritium removed from (R)-[1-^{3}H]ethanol. The theoretical basis is discussed in detail in chapter 3.

MATERIALS AND METHODS

Radiochemicals

Ethanol-1-^{14}C was purchased from New England Nuclear Corporation, Boston, Massachusetts and from Amersham-Searle Corporation, Arlington Park, Illinois. It was purified by methods previously described (Chapter four p. 4.3). Counting was performed on a Nuclear Chicago 727 scintillation counter. The total dpm in ^{3}H and ^{14}C were determined by internal standardisation.

Incubations

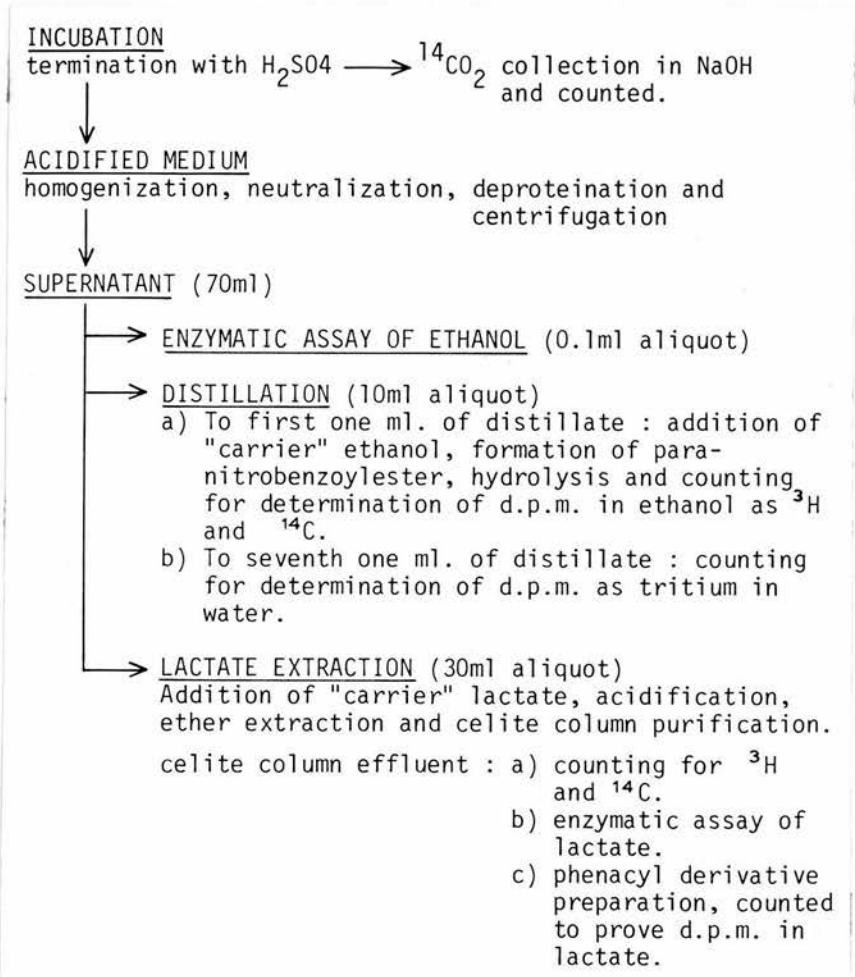
Four experiments were performed. In each experiment a flask containing liver slices suspended in medium was incubated with 1.25 uCi of either (R)-[1-^{3}H]ethanol or (S)-[1-^{3}H]ethanol. Identical control flasks were set up

(also containing either (R) or (S) isomer) from which the slices were omitted. 500 ml Erlenmeyer flasks were used each containing 30 ml of medium (Hastings et al. 1952). The composition of the medium in millimoles per litre was as follows: K^+ = 110, Mg^{++} = 20, Ca^{++} = 10, HCO_3^- = 40 and Cl^- = 130. Glucose and ethanol were both present at a concentration of 1 mg per ml. Ethanol-1-^{14}C (0.25-0.5 uCi) was added to all the flasks. Liver slices from 2 rats were randomly distributed into a pair of flasks each containing either (R) or (S) 1-tritiated ethanol. Sprague-Dawley rats (Charles River, Wilmington, Mass.) weighing 260-300 grams were used. They were fed ad libitum prior to killing by decapitation. Between 3.6 and 4.4 grams of slices were added to each flask. Paired flasks differed in weight of slices by no more than 0.1 gram.

The flasks were gassed for 10 minutes with 95% O_2/5% CO_2. They were stoppered and shaken at 37^o for 90 minutes. 3 ml of 2N H_2SO_4 was injected into each flask through a rubber inlet in the stopper. A similar volume of CO_2-free NaOH was then injected into a vial suspended from each stopper to collect evolved CO_2. In experiments 3 and 4 (Table 7.2) 32 mg of carrier acetaldehyde in aqueous solution was injected through the inlet into each of the flasks following the addition of sulphuric acid. Subsequent steps involved in processing the medium are outlined in Fig. 7.1.

The sealed flasks were stored for 12 hours to allow complete absorption of the $^{14}CO_2$ into the sodium hydroxide. The acidified medium with the slices in each flask was then cooled in ice and homogenised. The medium was then centrifuged

Fig. 7.1. THE ANALYSIS OF MEDIA FROM INCUBATIONS USING THE ENANTIOMERS OF [1-^{3}H] ETHANOL AS TRACERS



in a closed bottle at 4°. The supernatant was neutralised, deproteinated with 1 ml of 0.3 N $Ba(OH)_2$ and 1 ml of 5% $Zn(SO)_4$ and recentrifuged at 4°C. The concentrations of ethanol in an aliquot of the supernatant and in the initial medium were determined enzymatically (Bonnichsen and Theorell, 1951) and the quantity of ethanol taken up during the incubation was calculated. There was no disappearance of ethanol in the control flasks without added slices.

Distillation Procedure

In order to measure separately the radioactivity present in tritiated water and in ethanol at the end of incubation I devised the following method taking advantage of the fact that an ethanol and water mixture distil as an azeotropic mixture containing 96% ethanol. Thus if a mixture containing a small amount of ethanol is distilled most of the ethanol will be collected in the early distillate and the subsequent collection will be relatively free from alcohol. A 10 ml aliquot of each supernatant was made alkaline with NaOH (to prevent possible distillation of organic acids) and distilled. The first one ml of distillate was used to prepare the insoluble p-nitro benzoate ester of ethanol. The next five ml of distillate were discarded. The seventh ml was used to determine the dpm of tritium in water.

Counting of dpm in Ethanol

To the first one ml of distillate and to an aliquot of the initial medium, carrier unlabelled ethanol was added and the p-nitrobenzoate ester of ethanol was prepared (Henstock, 1933). The yellow precipitate was washed and dried. On dissolving in Aquasol it caused a high degree of quenching

rendering ratios of ^{3}H:^{14}C totally unreliable. This problem was surmounted by hydrolysing the ester and counting the ethanol thus regenerated. Hydrolysis was accomplished by refluxing in 1.N.NaOH. Ethanol was distilled from the resulting solution. Using this method the ^{3}H:^{14}C ratios in ethanol for post-incubation control flask media were identical with those of the labelled ethanol originally added to the flasks. This supported the validity of the method as a means of determining the dpm present in ethanol.

Calculation of Tritium taken up from Medium Ethanol

It was not possible to measure accurately the total dpm of both isotopes in medium ethanol at the end of incubation. This was because a quantitative collection of ethanol from the ester could not be achieved. Tritium uptake from the medium was therefore calculated as follows:

^{3}H uptake from medium = (initial total ^{3}H dpm in medium)-(final total ^{3}H dpm in medium).

= (^{3}H dpm in aliquot of control medium x volume correction factor).

- ($^{3}H/^{14}C$ ratio at termination x initial total ^{14}C dpm in medium x fraction of ethanol in medium at termination measured enzymatically).

This calculation assumes only that there is insignificant isotope discrimination between ^{14}C and ^{12}C so that the percentage uptake of ethanol and that of $[1\text{-}^{14}C]$ethanol is identical. In experiments subsequently performed in the same laboratory the p-nitrobenzoate esters were oxidised to $^{14}CO_2$ and $^{3}H_2O$ in an oxidiser. Uptake was then calculated directly and data so obtained have validated the above method.

Counting of Tritium in Water

An aliquot of the seventh ml of the distillate (v. supra) was counted to determine the total dpm present as ^{3}H and ^{14}C. The tritium present in tritiated water was estimated from the following formula:

^{3}H in $^{3}H_2O$ = (total ^{3}H dpm counted in final distillate) - (total ^{14}C dpm in distillate x ^{3}H:^{14}C ratio of post-incubation medium ethanol).

The ^{3}H:^{14}C ratio of ethanol used was obtained from para-nitrobenzoate hydrolysis as described above. This method assumes that all ^{14}C is present in alcohol and that all tritium is either present in alcohol or water. In the seventh ml of distillate about one quarter of the total dpm in tritium was present in ethanol.

Negligible tritium was present in $^{3}H_2O$ in the media from control flasks. $^{3}H_2O$ (obtained from New England Nuclear Corporation) was distilled in a control experiment. The specific activity of the initial and final distillates was identical. A similar finding was obtained by Simpson and Greening (1960). The total tritium incorporated into water was therefore calculated from the tritium in the aliquot of the distillate and the total volume of the supernatant.

Lactate Extraction

To another aliquot of each supernatant carrier lactate was added. The aliquot was acidified with H_2SO_4 and the lactate was extracted with ether. The lactate in the ether was isolated as its sodium salt and purified on a Celite column (Bernsteinand Wood, 1957). The lactate content of this final solution was measured enzymatically (Hohorst, 1965)

and an aliquot was counted. Lactate from the column was converted to its phenacyl derivative (Rather and Reid, 1919) which was recrystallised, washed and counted. The $^{3}H:^{14}C$ ratio of this approximated closely to that of the final solution confirming that the radioactivity measured was in lactate.

Acetaldehyde Isolation and Counting

In two experiments (numbers 3 and 4, Table 7.2) carrier acetaldehyde was added and an aliquot of the supernatant from each flask was distilled directly into dimedone reagent in a test tube. The tube was stoppered and warmed to complete the formation of dimetoacetaldehyde which was collected (see page 4.4) and counted for ^{14}C and ^{3}H. The quantities of ^{3}H and ^{14}C in acetaldehyde in the incubation medium with slices at the completion of incubation were calculated from the 32 mgs of acetaldehyde added as carrier and the weight of dimedone assayed for radioactivity. Negligible ^{3}H and ^{14}C radioactivity was present in dimetoacetaldehyde isolated from the control incubations. The quantity of acetaldehyde present in the slices and medium of each flask (post-incubation) was calculated from the total ^{14}C in acetaldehyde (post-incubation) and the molar specific activity of acetaldehyde-1-^{14}C formed. This was assumed to be the same as that of the ethanol-1-^{14}C incubated.

Counting of $^{14}CO_2$

The $^{14}CO_2$ absorbed into the sodium hydroxide was precipitated as $Ba^{14}CO_3$. The $Ba^{14}CO_3$ was weighed and then treated with H_2SO_4. The $^{14}CO_2$ evolved was collected in ethylenediamine in methylcellusolve (Kornblatt et al., 1964) and assayed for ^{14}C. Incorporation into $^{14}CO_2$ was calculated from the

specific activity of the $^{14}CO_2$ and the total quantity of CO_2 estimated to be present in the medium and gas phase of each flask.

Loss of Acetaldehyde

A control experiment showed that an insignificant amount of acetaldehyde was lost during the course of the incubation. [1-^{3}H] Acetaldehyde (5 mg) was injected into medium with slices immediately after acidification and without incubation. It was then incubated for 90 minutes and carrier acetaldehyde (32 mg) was added and the above procedure followed. Twenty-six mgs of precipitate were recovered. Its specific activity was such that the theoretical yield of dimetoacetaldehyde from 37 mg of acetaldehyde (257 mgs) would have contained 97% of the dpm added to the flask.

RESULTS

The results of the four experiments are presented in Tables 7.1 and 7.2. The statistical methods used are described in the legend of ~~Fig.~~ Tab 7.1. About one half of the medium ethanol measured enzymatically was taken up. The per cent uptake of tritium from (R)-[1-^{3}H] ethanol was similar to this value. (Table 7.1). This is reflected in the $^3H:^{14}C$ ratio in ethanol which remained approximately constant. (Table 7.2). In contrast the uptake of tritium from (S)-[1-^{3}H] ethanol was only slightly more than half the uptake of "cold" ethanol ($p<.01$, Table 7.1). This resulted in a consistent increase in the $^3H:^{14}C$ ratio following incubation (Table 7.2).

The total C^{14} yield in $^{14}CO_2$ was similar in flasks containing (R) and (S) isomers. This provides further

TABLE 7.1

METABOLISM OF (R) AND (S) 1-TRITIATED ETHANOLS WITH [1-^{14}C] ETHANOL BY RAT LIVER SLICES

	% Ethanol Uptake	% ^{3}H Uptake	% of ^{14}C to $^{14}CO_2$	% of ^{3}H taken up to :		
				Lactate	Water	Acetald.
(R)	50.5±2.9	49.3±2.6	3.95±0.86	12.4±3.9	68.35±8.1	0.057
(S)	46.5±2.3	29.8±2.5	4.38±0.8	2.6±0.33	62.55±3.2	0.55
n	4	4	4	4	4	2
Significance	N.S.	$p < 0.01$	N.S.	$p=0.014$	N.S.	

Results are expressed as the mean ± S.E.M. The number of experiments is denoted by n. Statistical significance was tested using the unpaired t test. The method of Mann and Whitney (1947) was used with the percentages of utilised tritium appearing in lactate which were not normally distributed.

TABLE 7.2

THE $^{3}H:^{14}C$ RATIOS IN ETHANOL AND ACETALDEHYDE IN INCUBATIONS USING (R) AND (S) 1-TRITIATED ETHANOL WITH $[1-^{14}C]$ ETHANOL

Experiment		Ethanol (initial)	Ethanol (final)	Acetaldehyde (final)
1	R	4.64	4.42	-
	S	4.65	7.46	-
2	R	4.64	4.63	-
	S	4.65	6.74	-
3	R	2.72	2.79	0.39
	S	2.53	3.27	3.29
4	R	2.72	3.0	0.43
	S	2.53	3.37	3.62

The $^{3}H:^{14}C$ ratios in ethanol are given at the beginning and completion of incubation and in acetaldehyde at completion only. Rat liver slices were incubated with either (S)$[1-^{3}H]$ethanol or (R)$[1-^{3}H]$ethanol in the presence of $[1-^{14}C]$ ethanol.

evidence for similar rates of ethanol oxidation in paired flasks. A mean value of 12.4 per cent of the tritium utilised (i.e. transferred from medium ethanol) appeared in lactate in flasks containing (R) 1-^{3}H ethanol. This exceeded by almost five times the proportion in flasks containing the (S) isomer ($p = 0.014$, ~~Fig~~ Tab. 7.1). However a similar percentage (about 60%) of tritium metabolised was recovered in water from both isomers.

A very small proportion (0.55%) of metabolised tritium appeared in acetaldehyde with flasks containing (S) 1-^{3}H ethanol as tracer. With (R) 1-^{3}H ethanol this proportion was one tenth of the above value. The $^{3}H:^{14}C$ ratios in acetaldehyde are presented in Table 7.2. With the (S) isomer the ratios approximate to those found in ethanol at the completion of incubation. In flasks containing (R)-[1-^{3}H] ethanol as tracer the ratio was very low in comparison with the alcohol. The acetaldehyde content of the final medium was calculated on the assumption that the molar specific activity of the acetaldehyde equals that of the [1-^{14}C] ethanol. This gave the following results: 3R = 52 µg, 4R = 55 µg, 3S = 30 µg and 4S = 43 µg.

DISCUSSION

In the flasks containing (S) 1-^{3}H ethanol the uptake of tritium was less than that of protium measured in the enzymatic ethanol assay. This differential uptake was reflected in an increase in the $^{3}H:^{14}C$ ratio of ethanol at termination (Table 7.2). Furthermore a similarly enriched $^{3}H:^{14}C$ ratio was present in acetaldehyde with the (S)-isomer in experiments 3 and 4. These findings are explicable on

the basis of a primary kinetic isotope effect involving the oxidation of [1-^{3}H] acetaldehyde. If there were no kinetic isotope effects the ratios of ^{3}H:^{14}C in acetaldehyde and ethanol would show no change throughout the experiment. The increase in the ratio of acetaldehyde reflects a discrimination of tritium versus protium in the oxidation of tracer quantities of [1-^{3}H]acetaldehyde. The increase of the ^{3}H:^{14}C ratio in ethanol accrues from the known reversibility of the oxidation of ethanol to acetaldehyde. In Chapter 9 evidence is presented for this reversibility in experiments using [2-^{3}H] sorbitol as tracer in an identical liver slice preparation. An alternative explanation is that the presence of tritium in the (S) position induces a significant secondary kinetic isotope effect in the oxidation of ethanol to acetaldehyde. However in this instance an increase in the ratio of ^{3}H:^{14}C in acetaldehyde (in comparison with the ratio in the initial alcohol) would not have been observed. In experiments 3 and 4 using (R)-[1-^{3}H]ethanol as tracer a slight increase in the ^{3}H:^{14}C ratio in alcohol was observed. This may represent a minor degree of primary kinetic isotope effect in the oxidation of (R)-[1-^{3}H]ethanol. The ^{3}H:^{14}C ratios in acetaldehyde using the (R) isomer are very low being 0.39 in experiment 3 and 0.43 in experiment 4. In theory acetaldehyde derived from (R)-[1-^{3}H]ethanol should contain no tritium at all since all known oxidative systems remove this hydrogen atom (see Chapter 2, 5 and 6). The presence of this small amount of tritium in acetaldehyde probably results from isotope in the (S) position of (R)-[1-^{3}H]ethanol. This ectopic tritium combined with the

primary kinetic isotope effect postulated in the oxidation of acetaldehyde (v. supra) could explain the presence of tritium in acetaldehyde from the (R) isomer. The significant kinetic isotope effect observed with (S)-[1-^{3}H]ethanol does not by itself invalidate this radio-chemical as a tracer. Thus it should still be possible by examining the fate of tritium taken up from the medium to draw meaningful conclusions regarding the disposal of transferred isotope in the cell.

However when the fate of that tritium taken up from the medium is examined it is seen to be quite different with the two enantiomers. In tritiated water the fractions counted are similar, while in lactate the proportion derived from (R)-[1-^{3}H]ethanol exceeds that from the (S) isomer by almost five fold. The most likely explanation for this difference is that acetaldehyde is not oxidised in the cytoplasm. The following are unlikely alternative explanations of these data:

1. There are two pools of NADH in the cytoplasm. Convincing evidence for this possibility is lacking.
2. The enzyme responsible for acetaldehyde oxidation exhibits B-type stereospecificity. Aldehyde dehydrogenase was examined by Levy and Vennesland (1927) and found to be A-type.
3. Acetaldehyde is not oxidised by a NAD-linked dehydrogenase.

The alternative oxidative mechanism for acetaldehyde are discussed on pages 1.22 to 1.23. It is considered very unlikely that these mechanisms participate in the oxidation of acetaldehyde during ethanol metabolism <u>in vivo</u>. The transfer of tritium to $NADP^{+}$ and not NAD as coenzyme during the oxidation of acetaldehyde could possibly explain the different distribution of tritium from (S)-[1-^{3}H] ethanol.

Tottmer et al. (1973) demonstrated that NADP could serve as coenzyme to the Type II aldehyde dehydrogenase isolated from rat liver (v. infra). However the Km for acetaldehyde using $NADP^+$ with this enzyme is too high (0.5 to 0.7 mM) to be implicated in the oxidation of acetaldehyde at the levels found in vivo.

4. If the oxidation of $[1-^3H]$ acetaldehyde involved a marked primary kinetic isotope effect it is conceivable that tritium could be sequestered in acetaldehyde and therefore not be transferred to lactate. In experiments 3(S) and 4(S), however it was shown that less than 1% of utilised tritium was present in acetaldehyde. Furthermore the occurrence of similar proportions of utilised tritium in water is against this possibility.

The fate of transferred tritium from (S)-$[1-^3H]$ ethanol therefore strongly suggests that acetaldehyde is not oxidised in the cytosol. Evidence implicating the mitochondria as the major site of oxidation is presented in the next paragraph.

At this juncture it is convenient to review recent evidence concerning the subcellular site of acetaldehyde oxidation. When the work described in this chapter was carried out the consensus of opinion favoured the view that oxidation of acetaldehyde occurred in the cytoplasm (see Chapter 1). On this basis $[1-^3H]$ acetaldehyde (produced in the cell from (S) $[1-^3H]$ ethanol) would act as a suitable labelled hydrogen donor as described in Chapter 3 p. 3.4 However Marjansen in 1972 performed a careful fractionation of aldehyde dehydrogenase in rat liver and located 80% of such activity in the mitochondria. This mitochondrial enzyme

had a low Km of 10 uM. Grunnet (1973) found a similar quantitative localisation of NAD-dependent aldehyde dehydrogenase activity in the same species. His data indicated that the mitochondrial enzyme may have two separate Km values of 0.1 uM and 1 mM. Tottmar et al. (1973) also in the rat demonstrated the presence of two separate aldehyde dehydrogenases. Enzyme I was located exclusively in mitochondria and had a low Km for acetaldehyde (10 uM). Enzyme II situated in mitochondrial and microsomal fractions had a much higher Km (0.9-1.7 mM) and reacted with NADP as well as NAD. Deitrich (1971) found most of the aldehyde dehydrogenase activity in the mitochondrial fraction. He also showed that phenobarbitone administration to genetically selected rats increased aldehyde dehydrogenase activity in supernatant only. This provides good evidence for the independent nature of these two enzymes. From these recent studies it may be concluded that there is more than one NAD-dependent aldehyde dehydrogenase in the ~~cytoplasm~~ cell. Eighty per cent is located in the mitochondria and has a stronger affinity for acetaldehyde.

The relevance of these recent sub-cellular localisation studies to the oxidation of acetaldehyde has been confirmed by the work of Lindros et al. (1974) and Parilla et al. (1974). Lindros et al. (1974) have examined the oxidation of ethanol in the in vitro perfused rat liver preparation. The intracellular concentration of ethanol was determined via spectrophotometric measurement of the Catalase-H_2O_2 intermediate, Compound I. Changes in the reduction state of flavine and pyridine nucleotides were measured by surface fluorometry and compared with changes in the ratios of

lactate to pyruvate and of β-hydroxybutyrate to acetoacetate in the perfused liver effluent. Observations made during ethanol and acetaldehyde infusions showed that at acetaldehyde concentrations below 0.4 mM oxidation occurs in the mitochondrial compartment. With higher acetaldehyde concentrations there was evidence of oxidation in the cytoplasmic phase. Parilla et al. (1974) examined acetaldehyde oxidation in perfused rat liver and in suspensions of isolated murine hepatocytes. The influence of transaminase inhibitors on acetaldehyde utilisation and the effects of acetaldehyde on the reduction state of the cytosolic and mitochondrial pyridine nucleotides was examined in detail. Their data agreed with Lindros et al. (1974) in regard to the site of acetaldehyde oxidation at different concentrations. Furthermore they showed that reducing equivalents generated in the cytosol were transported to the mitochondria by the malate-aspartate shuttle at concentrations of acetaldehyde above 0.4 mM.

Steady state concentrations of acetaldehyde below 0.2 mM have been assayed in blood (Lundquist and Wolthers, 1958; Truitt, 1970; Korsten et al., 1975) and in liver perfusate (Williamson et al., 1969; Lindros et al., 1972) during ethanol oxidation. It therefore follows that during ethanol metabolism in vivo acetaldehyde will be oxidised predominantly in the mitochondria. The physiological role of the cytoplasmic enzyme which cannot be involved at these concentrations is difficult to understand. If the amount of acetaldehyde found at termination in experiments 3 and 4 (Table 7.2) was present in an estimated 3 ml of cell water the intracellular concentration range would be 0.23 to 0.42 mM.

It seems likely that some acetaldehyde diffused into the medium and therefore the actual intracellular concentration would be even lower than these values. In my experiments therefore acetaldehyde concentrations are in the range normally found during ethanol metabolism. This supports the validity of this liver slice preparation as a model for oxidation *in vivo*.

I conclude therefore that the distribution of utilised tritium in flasks containing (S)-[1-^{3}H] ethanol indicates that the oxidation of [1-^{3}H] acetaldehyde takes place predominantly in the mitochondria. From the different proportions counted in lactate it appears that at most one quarter to one fifth of oxidation is occurring via a cytoplasmic NAD-linked pathway. This estimate assumes that all alcohol in rat slices is metabolised to acetaldehyde via the NAD-linked enzyme alcohol dehydrogenase. If pathways that do not produce NADH such as catalase or MEOS are operating an underestimation of the proportion of acetaldehyde oxidised in the cytosol would result. However evidence will be presented in Chapter 9 that the contribution of such pathways in the rat is quantitatively insignificant at the ethanol concentration used in the present experiments. The present experimental data extend the subcellular localisation studies to the intact cell without recourse to enzyme inhibitors. They confirm the conclusions of Lindros *et al.* (1974) and Parilla *et al.* (1974) arrived at with quite different experimental approaches. The present experiments which were designed to trace the pathways of ethanol oxidation *in vivo* have given information about acetaldehyde disposal. This finding may not hold for other species

because the cytoplasmic enzymes extracted from bovine (Kacker, 1949; Deitrich et al., 1962) equine (Feldman and Weiner, 1972) and human liver (Blair and Bradley, 1969; Kraemer and Deitrich, 1968) have Km values for acetaldehyde that are 2 to 3 orders of magnitude lower than in the rat.

In the study of Rognstad and Clark (1974) tritiated tracers were incubated with isolated hepatocytes obtained from fasting rats. Known substrates of mitochondrial dehydrogenases were found to give a characteristic pattern of a lower specific yield of tritium in glucose and a higher percentage of this isotope at the C-6 position of glucose than cytosolic substrates. It was concluded on the basis of experiments using (S)-[1-^{3}H] ethanol as tracer that acetaldehyde is oxidised by a mitochondrial dehydrogenase enzyme. The study supports my own findings but their experimental conditions were such that grave reservations must be held in regard to any conclusions. The initial concentrations of medium ethanol were in the range 20 to 200 nanomol/ml and medium lactate was 8 μmol/ml. Thus the ethanol concentration is incomparably lower than plasma levels assayed in animals after even the smallest amount of ethanol ingestion. In addition to its irrelevance to the whole animal situation this minute quantity of alcohol would be mostly metabolised as evidenced by the high isotopic recovery in water. Furthermore on a molar basis the amount of lactate exceeded alcohol by 400 fold. This could have a significant effect on the mode of ethanol oxidation via redox changes. In my experiments a "physiological" ethanol concentration was chosen in order to ensure complete

saturation of pathways and achieve steady state conditions during the course of the incubation.

CHAPTER 8: DETERMINATION OF THE STEREOSPECIFICITY OF SORBITOL DEHYDROGENASE

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION

INTRODUCTION

From the data presented in the previous chapter it is clear that (S)-[1-^{3}H] ethanol is not a suitable tritium donor with which to compare the fate of isotope removed from (R)-[1-^{3}H] ethanol. Its unsuitability accrues from the evidence presented that the tritium is removed in a subcellular compartment other than the cytoplasm. In order to apply the model outlined in Chapter 3 it was necessary to find an alternative donor. Such a compound would have to reduce NAD during its cytoplasmic oxidation by only one reaction. Furthermore it would have to be metabolised primarily in the liver and as an absolute requirement should be an A-type enzyme in transferring its hydrogen to NAD. A potentially suitable compound was [2-^{3}H] sorbitol.

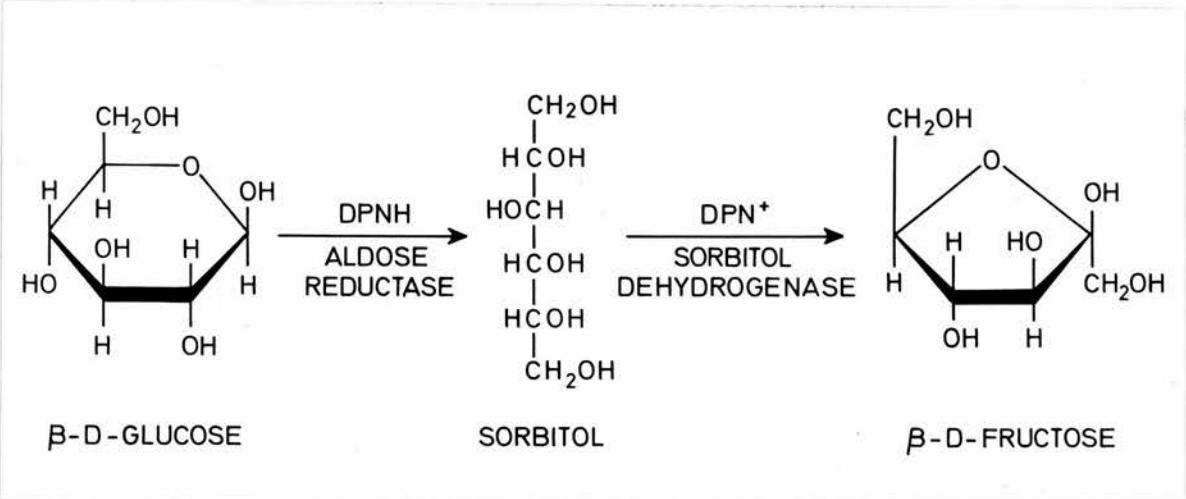
Sorbitol is metabolised almost completely in the liver (Todd et al., 1939) via the cytoplasmic enzyme sorbitol dehydrogenase to fructose (Fig. 8.1). In this oxidation it donates hydrogen removed from position 2 to the NADH pool. The stereospecificity of sorbitol dehydrogenase was unknown and prompted the experiments described in this chapter. Three experimental approaches were devised in one of which the hydrogen atom was labelled with tritium. In two others the transferred hydrogen atom was completely replaced by deuterium.

MATERIALS AND METHODS

Materials

[1-^{2}H]-D-Glucose and sodium borodeuteride both with a reported minimum purity of 98% deuterium and [^{2}H4] acetaldehyde with a reported minimum purity of 99% deuterium

Fig. 8.1. THE ENZYMES OF THE POLYOL PATHWAY



Erratum: The coenzyme of aldose reductase is TPN (NADP) and not DPN (NAD) as shown.

Glucose may be reduced to sorbitol by aldose reductase. The oxidation of sorbitol is catalysed by sorbitol dehydrogenase in the cytoplasm to yield fructose and N.A.D.H.

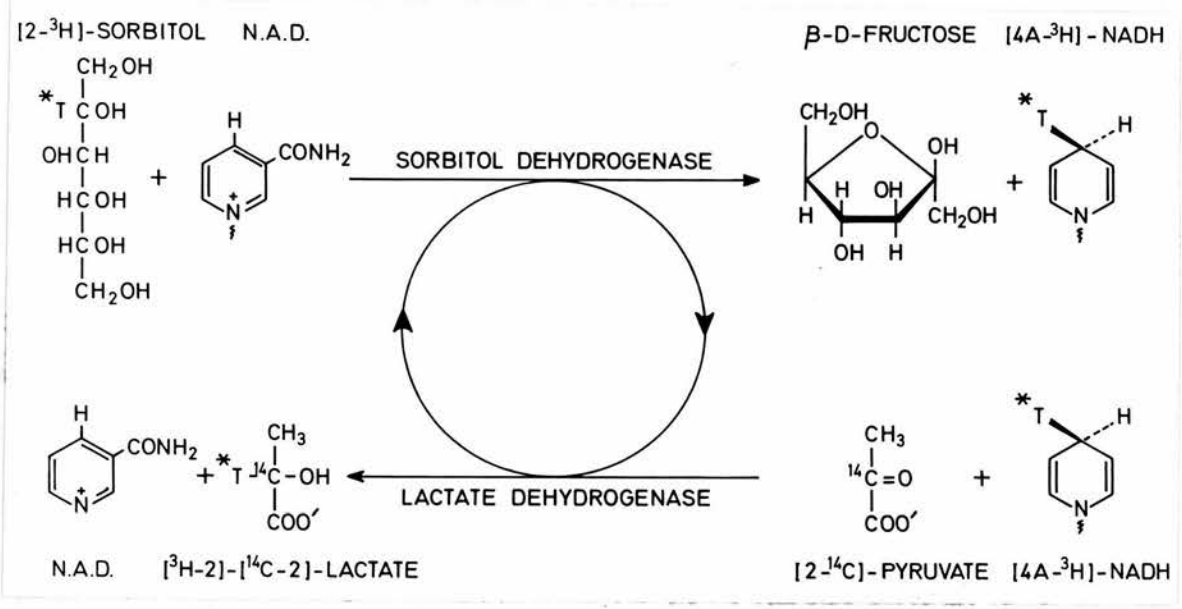
were purchased from Merck and Co. Inc. St. Louis, Missouri. Sodium (~~R,S~~)-[2-^{14}C] - Pyruvate (NEC-256) was purchased from New England Nuclear Corporation and was further purified by elution from a Dowex-1-X1 (sulphate form) column. [2-^{3}H]-D-Glucose (NET 238) from the New England Nuclear Corporation was reduced to [2-^{3}H] sorbitol using sodium borohydride (Abdel-Akher et al., 1951) and purified by paper chromatography. The following enzymes were purchased from Sigma Company: sorbitol dehydrogenase (S1128), lactate dehydrogenase (L2625), aldehyde dehydrogenase (A 6758), glucose dehydrogenase (G 5625) and glucose oxidase (NG 6125).

Method 1. The Stereospecificity of sorbitol dehydrogenase using lactate dehydrogenase.

The underlying reaction is shown in Fig. 8.2. Oxidation of [2-^{3}H] sorbitol to fructose would produce NADH labelled with tritium in the A position if this enzyme exhibited A-type stereospecificity. This would subsequently be transferred by the coupled reaction to lactate since lactic dehydrogenase is an A-type enzyme (Loewus et al., 1953). ^{14}C-labelled pyruvate was used to assess the tritium content per mole of lactate so formed.

In a stoppered 25 ml Erlenmeyer flask 0.8 mg of sorbitol dehydrogenase, 0.25 mg of lactate dehydrogenase, 10 mg of NAD, 150 mg of sodium (~~R,S~~) pyruvate, 0.3 uCi of sodium (~~R,S~~)-[2-^{14}C] pyruvate, 2 mg of sorbitol and 0.3 uCi of 2-^{3}H -sorbitol were incubated at 37^{o}C in 4.7 ml of phosphate buffer 0.05M, pH 8. After 2 hours the reaction was terminated by the method of Somogyi (1945). Following centrifugation lactate was extracted from the supernatant with ether after

Fig. 8.2. THE STEREOSPECIFICITY OF SORBITOL DEHYDROGENASE USING LACTATE DEHYDROGENASE



This shows the transfer of tritium that would occur if sorbitol dehydrogenase was an A-type enzyme.

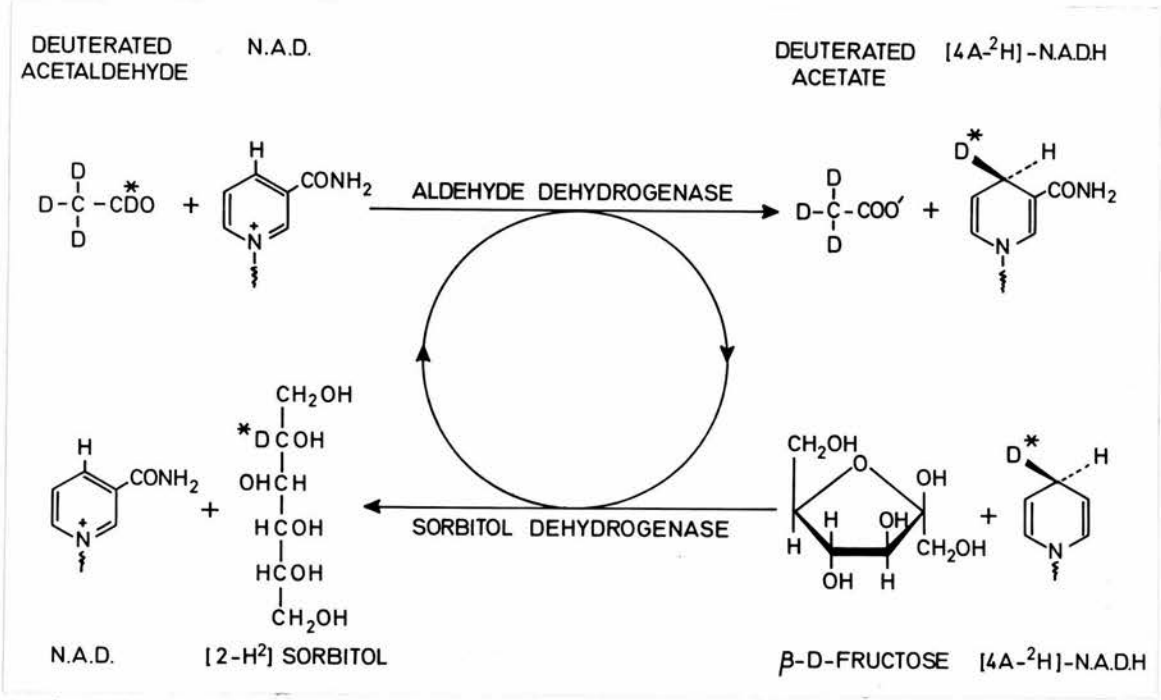
addition of 0.5 ml of 10N H_2SO_4 (Bernstein and Wood, 1957). The lactate was then separated from pyruvate with a Dowex-1-formate column (Busch et al., 1952) and the fractions containing lactate were pooled and steam distilled. 48 mg of lithium lactate was added as carrier and it was further purified on a celite column (Swim and Krampitz,1954). The lactate containing fractions were pooled and after evaporation to dryness were taken up in 0.5 ml of water and converted to the crystalline phenacyl derivative (Kather and Reid, 1919). A known weight of dried crystals were dissolved in aquasol and counted for ^{3}H and ^{14}C.

Method 2. The Stereospecificity of sorbitol dehydrogenase using aldehyde dehydrogenase.

The coupled enzymatic reaction is shown in Fig. 8.3. Aldehyde dehydrogenase an A-type enzyme (Levy and Vennesland, 1957) produces NADH labelled with deuterium in the A position. If sorbitol dehydrogenase is also an A-type enzyme it should reduce fructose to $[2\text{-}^{2}H]$ sorbitol demonstrable by gas chromatographic - mass spectrometry.

In a stoppered 125 ml Erlenmeyer flask 2 mg of sorbitol dehydrogenase, 12 mg of aldehyde dehydrogenase, 100 mg of NAD, 160 mg of fructose, 3 ul of mercaptoethanol and 9.6 mg of $[^{2}H4]$ acetaldehyde were incubated in 40 ml of potassium phosphate buffer, pH 7.0, 80 mM at 37°. After 2 hours the reaction was stopped by the addition of 0.5 ml of 0.3N $Ba(OH)_2$ and 0.5 ml of 5% $ZnSO_4$ (Somogyi, 1945). The suspension was centrifuged and the supernatant deionized by passage through a column of mixed bed ion exchange resin (Amberlite MB-3, Mallinkrodt Chemical Works, St. Louis,

Fig. 8.3. THE STEREOSPECIFICITY OF SORBITOL DEHYDROGENASE USING ALDEHYDE DEHYDROGENASE



This shows the transfer of deuterium that would occur if sorbitol dehydrogenase was an A-type enzyme.

Missouri). The effluent was reduced by evaporation and applied to Whatman preparative 3 MM paper, and chromatography performed using an N-butanol:ethanol:water system (by volume 52:32:18) which separated fructose from sorbitol (Putnam, 1957). Sorbitol localised by guide spots was eluted, and an aliquot assayed for sorbitol content (Williams-Ashman, 1965). The fructose content was determined (Roe et al., 1949) in another aliquot and found to be negligible. The remainder of the solution was lyophilised to dryness.

The deuterium content of the sorbitol in this residue was assayed by Dr. Peter D. Klein of the Argonne National Laboratory, Argonne, Illinois. A gas chromatograph - mass spectrometer - accelerating voltage alternation system was used (Klein et al., 1972). The sorbitol was converted to its hexacetate (Lenhardt and Winzler, 1968) prior to gas chromatography on a 1 mm x 122 cm glass column packed with 1% SP-525 on 100/200 mesh Supelcoport. Column temperature was 180° ; injection and manifold temperatures were 250° . The ion source was operated at 150° , with the electron energy at 70 ev. The mass spectrum exhibits a base peak at m/e 115 ($C_5H_7O_3$) (Figure 8.4). [2-^{2}H] sorbitol, prepared by reduction of unlabelled fructose and sodium borodeuteride (Abdel-Akher et al., 1951) was used for reference. Abbreviated spectra of the labelled and unlabelled compounds are shown in Figure 8.5. The quantity of deuterium incorporated was measured as the increase in the ratio of ions at m/e 116/115.

Method 3. The Stereospecificity of sorbitol dehydrogenase using glucose dehydrogenase

The coupled enzymatic reaction is depicted in Fig. 8.6.

Fig. 8.4. MASS SPECTRUM OF SORBITOL HEXAACETATE

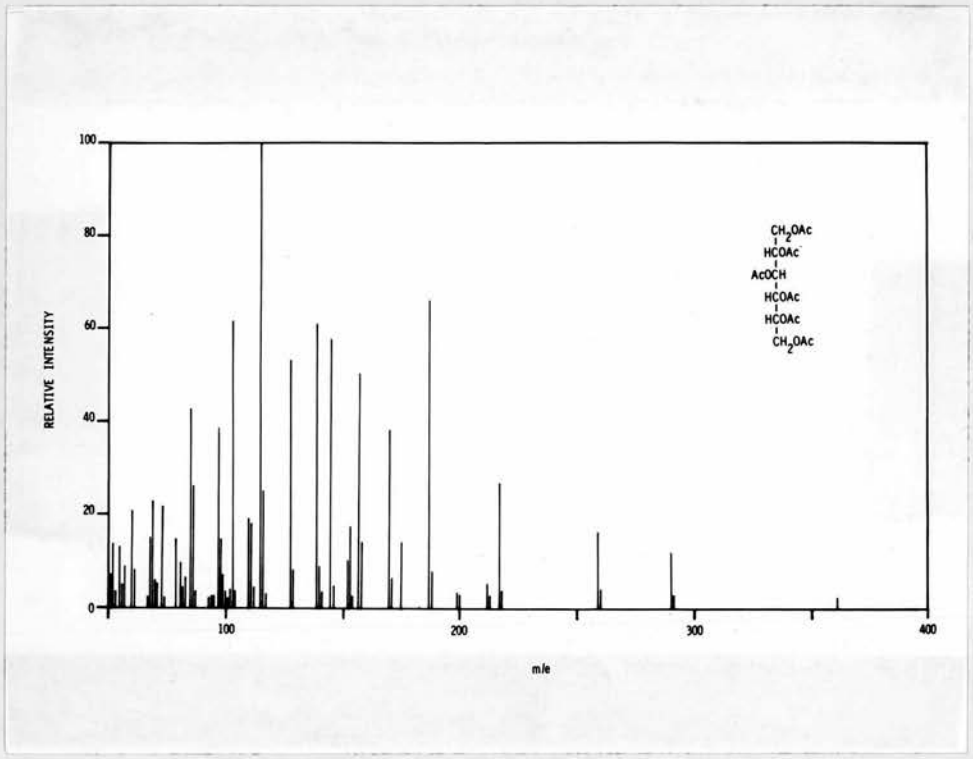


Fig. 8.5. PARTIAL MASS SPECTRA OF SORBITOL HEXAACETATE (A) AND [2-^{2}H] SORBITOL HEXAACETATE (B)

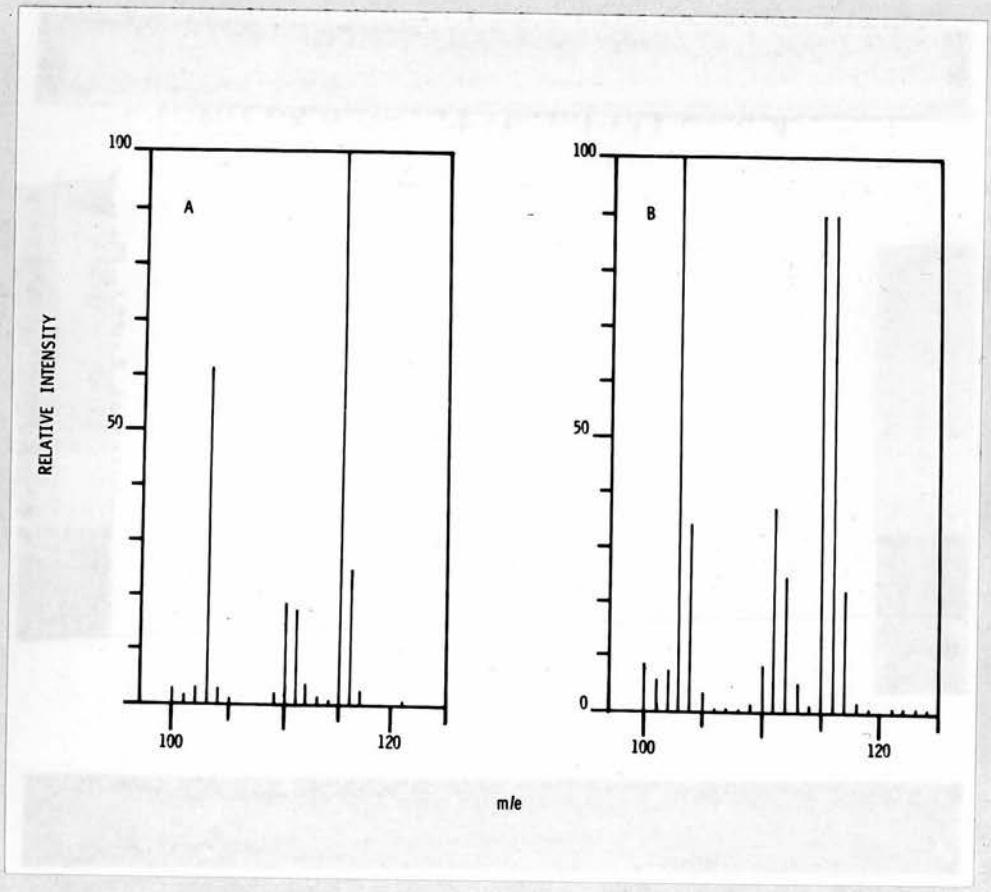
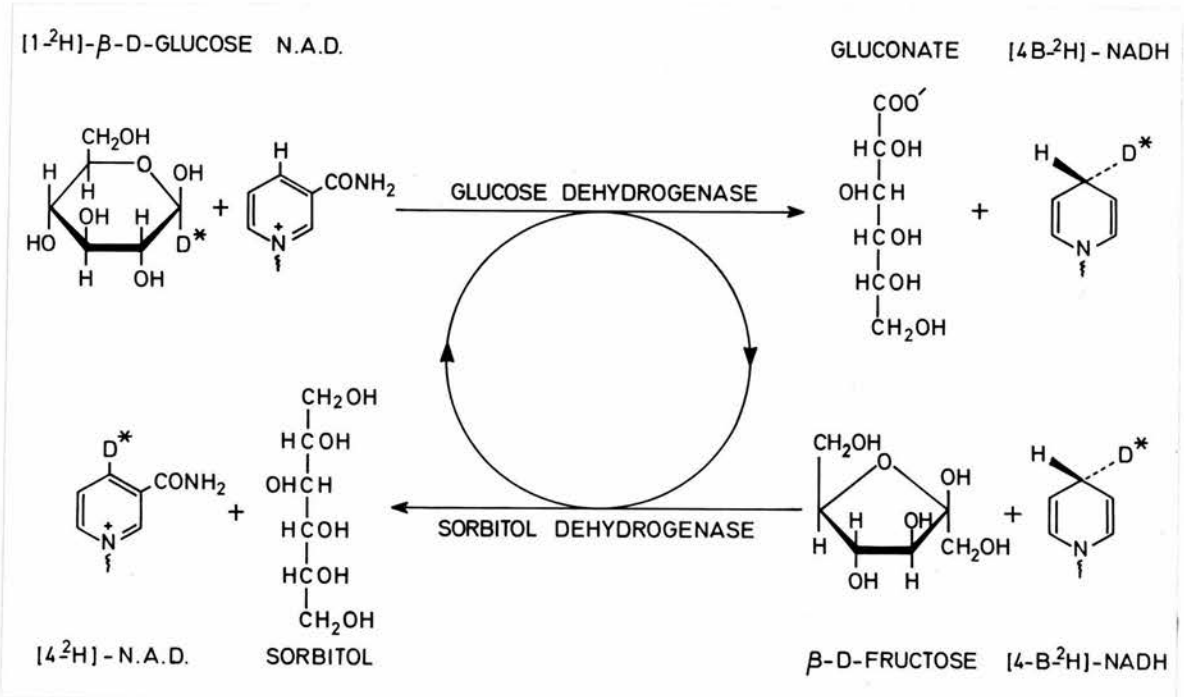


Fig. 8.6. THE STEREOSPECIFICITY OF SORBITOL DEHYDROGENASE USING GLUCOSE DEHYDROGENASE



This shows the transfer of deuterium that would occur if sorbitol dehydrogenase was an A-type enzyme.

Glucose dehydrogenase a B-type enzyme (Levy et al., 1956) forms $[4B\text{-}^2H]$-NADH during the oxidation of $[1\text{-}^2H]$-β-D-glucose to gluconate. If sorbitol dehydrogenase is also a B-type dehydrogenase the deuterium atom should be transferred to the C-2 position of sorbitol during reduction of fructose. If on the other hand it is an A-type enzyme deuterium will remain attached to NAD and sorbitol will contain no deuterium.

In a stoppered 10 ml Erlenmeyer flask 20 mg of glucose dehydrogenase, 0.3 mg of sorbitol dehydrogenase, 160 mg of NAD, 200 mg of fructose and 200 mg of $[1\text{-}^2H]$ glucose were incubated in 5 ml of 200mM phosphate buffer, pH 7.0 at 37°. After four hours the reaction was terminated by the addition of 0.5 ml of 0.3N $Ba(OH)_2$ and 0.5 ml of 5% $ZnSO_4$. The reaction mixture was centrifuged and the supernatant deionized on a mixed bed ion exchange column, concentrated and chromatographed on Whatman 3 MM paper using an n-butanol:glacial acetic acid:water system (by volume 50:25:25) (Fink et al., 1963) with good separation of glucose from sorbitol and fructose. The sorbitol and fructose areas, which were identified by guide spots overlapped. They were eluted with water and the eluate was incubated with glucose oxidase in phosphate buffer, pH 5.6, to convert any residual glucose to gluconic acid. The incubation was terminated with 0.3 N $Ba(OH)_2$ and 5% $ZnSO_4$, and the supernatant obtained after centrifugation was passed through another mixed bed exchange column to retain any gluconic acid. The eluate was concentrated and chromatographed in the butanol:ethanol:water system (Putnam, 1957) with isolation of the sorbitol and its analysis for deuterium content performed as described for method 2.

RESULTS

The results of the experiment performed by Method 1 are shown in Table 8.1. If sorbitol dehydrogenase was an A-type enzyme the ratio of the specific activities on a molar basis would be equal to the $^{3}H:^{14}C$ ratio of the isolated lactate. The ratio of 0.694 falls short of unity. This is explicable on a basis of isotopic discrimination ~~for~~ against tritium. If the enzyme exhibited B-type stereospecificity only minimal quantities of tritium, far less than those observed, would be counted in the isolated lactate. From the total ^{14}C content in lactate it was possible to calculate that 44% of the sorbitol had been oxidised to fructose.

The data from 2 pairs of experiments performed using deuterium are contained in Table 8.2. When aldehyde dehydrogenase was used as reference enzyme one atom of deuterium was incorporated per molecule of sorbitol formed. With glucose dehydrogenase minimal amounts of deuterium were present in isolated sorbitol.

DISCUSSION

These experimental data obtained by three different methods prove conclusively that sorbitol dehydrogenase is an A-type enzyme. The reference enzymes used in the coupled reactions were selected from the published data of their stereospecificity and electrode potential (E_o'). Incubation conditions were chosen on the basis of the Km values and pH optima etc. of the enzymes. In Method 1 using tritium as a label the experimental data were influenced by isotopic discrimination. In Methods 2 and 3 this was obviated by complete replacement of hydrogen with deuterium thereby

TABLE 8.1

STEREOSPECIFICITY OF THE SORBITOL DEHYDROGENASE CATALYSED REACTION USING LACTIC DEHYDROGENASE

Specific Activity of $[2\text{-}^{14}C]$ pyruvate in dpm/umole	(P)	375
Specific Activity of $[2\text{-}^{3}H]$ sorbitol in dpm/umole	(S)	45,892
Expected Ratio if sorbitol dehydrogenase is A-type	(S/P)	122
Observed ratio of isolated lactate		84.8
Ratio: Observed/Expected		0.694

TABLE 8.2

STEREOSPECIFICITY OF THE SORBITOL DEHYDROGENASE CATALYSED REACTION USING ALDEHYDE DEHYDROGENASE AND GLUCOSE DEHYDROGENASE

Enzyme	Sorbitol Isolated (mg)	Atoms ^{2}H Incorporated per Molecule
Aldehyde Dehydrogenase		
Experiment 1	4	0.99
Experiment 2	16	1.04
Glucose Dehydrogenase		
Experiment 1	3	0.02
Experiment 2	3	0.03

eliminating any competitive phenomena.

Rognstad and Clark (1974) examined the distribution of tritium in water and glucose from [2-^{3}H] sorbitol in isolated liver cells. From the observed distribution they concluded that sorbitol dehydrogenase appeared to be type B. The same author (Rognstad 1974,a) recanted this view after a more direct examination of the enzyme's stereospecificity. In a method involving isolation of the coenzyme and using tritium as label he obtained data consistent with an A-type stereospecificity. The recovery of tritium was 76% of the theoretical amount; a similar proportion was recovered in Method I a reflection in both cases of some isotopic discrimination against tritium. Alizade et al. (1974) has also recently confirmed that sorbitol dehydrogenase is an A-type enzyme.

The stereospecificity of sorbitol dehydrogenase from sheep liver (used in the present study) and from the rat should be identical. This lack of species variation is a constant finding to which no exceptions have been reported. For example alcohol dehydrogenases obtained from yeast (Fisher et al., 1953), equine liver (Levy et al., 1957) and Pseudomonas (Vennesland, 1955) all exhibit A-type stereospecificity. Krakow et al. (1963) have discussed the correlation of type of action with stereospecificity. There is some association of B-type stereospecificity with enzymes acting on carbohydrates and of A-type stereospecificity with enzymes oxidising the α-hydroxy acids. In general enzymes acting on smaller substrates exhibit A-type stereospecificity.

The demonstration of A-type stereospecificity with sorbitol dehydrogenase fulfilled an absolute requirement for its use as a tritium donor to investigate ethanol metabolism in liver slices. These experiments are described in the next chapter.

CHAPTER 9: QUANTIFICATION OF THE PATHWAYS OF ETHANOL OXIDATION IN RAT LIVER

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION

POSSIBLE DEVELOPMENTS OF THE ISOTOPIC METHOD

INTRODUCTION

The experiments described in this chapter were undertaken to quantify the pathways of ethanol oxidation in rat liver slices. $[2\text{-}^3H]$ Sorbitol has been used as labelled hydrogen donor in accordance with the scheme set out in chapter 3. The evidence validating this labelled substrate may be summarised as follows:

1. Sorbitol is metabolised almost exclusively in the liver (Todd *et al.*, 1939; Adcock and Gray, 1957; Wick and Drury, 1951) therefore enabling extrapolation of the method ultimately to the whole animal.

2. Sorbitol is oxidised by the cytoplasmic A-type NAD-linked enzyme sorbitol dehydrogenase (Chapter 8).

3. The oxidation of sorbitol is generally considered to be irreversible and to occur exclusively via sorbitol dehydrogenase. Isotopic evidence supporting this view will be presented in this chapter.

4. Sorbitol and ethanol oxidation appear to proceed via similar mechanisms. Isselbacher and Krane (1961) have shown similar redox changes during the oxidation of these two substrates by liver. Ethanol inhibited the oxidation of sorbitol by more than 30% (Verron, 1965) and sorbitol delays the oxidation of ethanol to a similar extent (Hillbrom, 1970). Finally Hillbrom and Lindros (1971) have presented evidence that this inhibition is mediated via competition for free NAD^+.

5. Sorbitol dehydrogenase activity in rat liver is not influenced by ethanol ingestion (Hillbrom and Pikkarainen, 1970).

The experiments described in this chapter are similar to those detailed in chapter 7 and differ mainly in the use of $[2\text{-}^3H]$ sorbitol instead of (S)-$[1\text{-}^3H]$ethanol as labelled

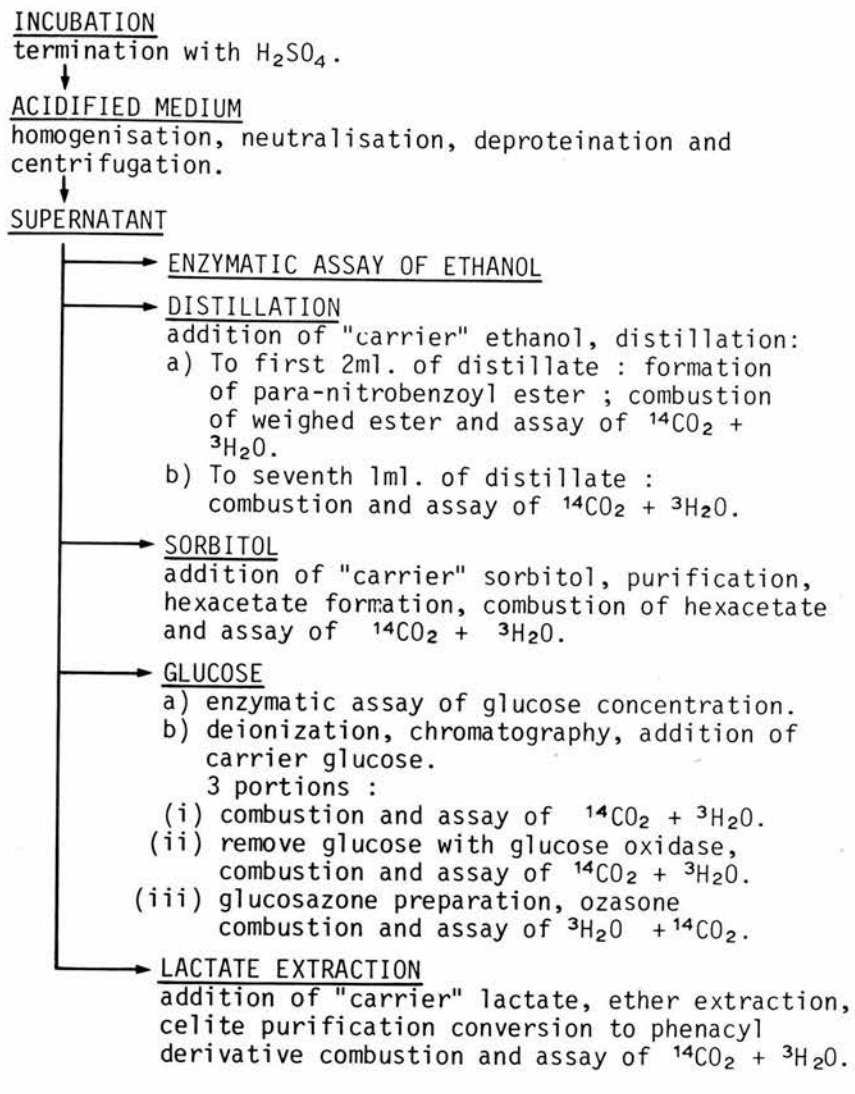
hydrogen donor. Other differences are apparent from a comparison of Figures 9.1 and 7.1 which outline the major steps involved in the two series of experiments. The present series of experiments have utilised the Packard 306 oxidiser to combust known weights of compounds with quantitative recovery of water and carbon dioxide. Thus radioactivity in tritium and ^{14}C can be counted separately avoiding the inaccuracies of ^{3}H:^{14}C ratio determinations. In addition carrier chemicals may be added in excess before the preparation of pure insoluble derivatives. By combusting a known weight of such a derivative it is possible to calculate the total dpm present in specific compounds present in the medium. I was involved directly only at the onset of the series of experiments described in this final chapter. The full series is included in this thesis because of my role in the practical implementation of the isotopic method. The data herein therefore represent the successful application of the theoretical model outlined in chapter 3.

MATERIALS AND METHODS

Radio-chemicals and Assay of Radioactivity

(R)-[1-^{3}H]Ethanol was prepared and purified as described in chapter 4. [1-^{14}C]Ethanol was purchased from New England Nuclear Corporation, Boston, Massachusetts and purified by the same procedure. [2-^{3}H]Sorbitol was prepared and purified as described in chapter 8. [2-^{14}C]Sorbitol was prepared by an identical method using [2-^{14}C] glucose (purchased from New England Nuclear Corporation) as starting material. Radio-active samples containing tritium and ^{14}C were combusted to $^{14}CO_2$ and $^{3}H_2O$ which were counted separately.

Fig. 9.1. THE ANALYSIS OF MEDIA FROM INCUBATIONS USING (R) -[1-^{3}H] ETHANOL AND [2-^{3}H] SORBITOL AS TRACERS



All counts were converted to dpm by internal standardisation.

Animals

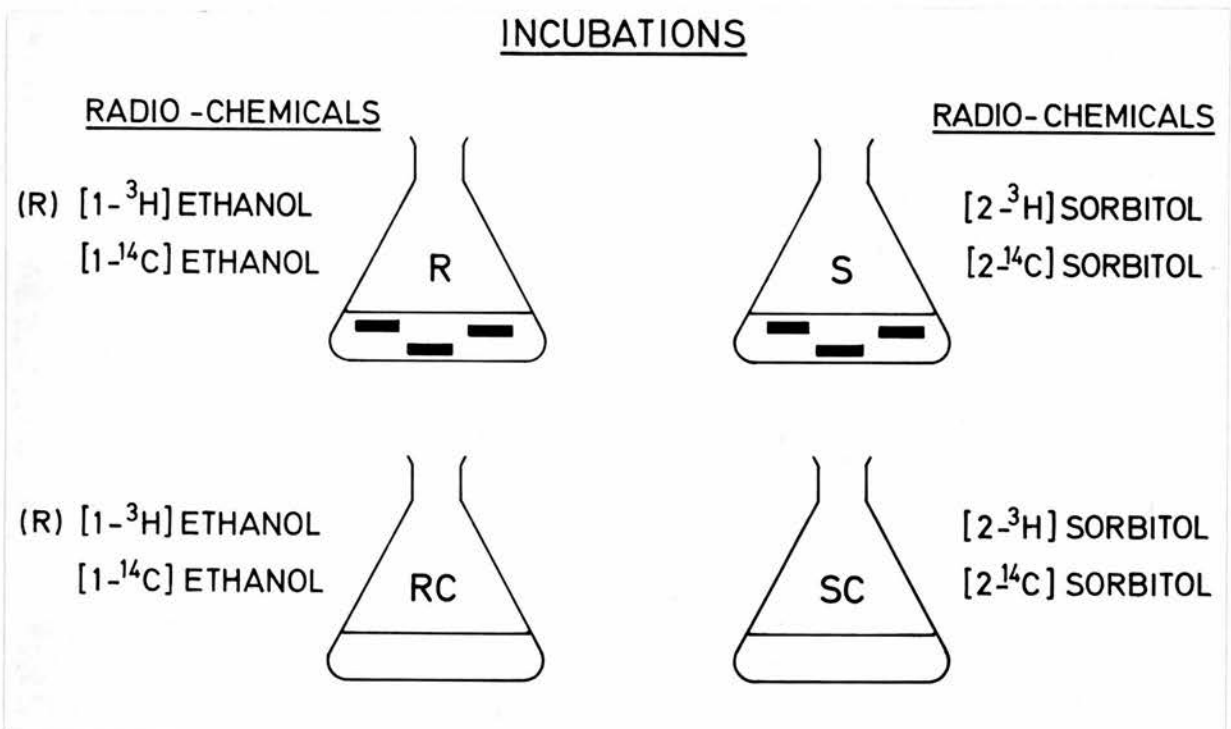
A group of Sprague-Dawley female rats (Charles River, Wilmington, Mass.) weighing 225 to 300 grams were fed on stock chow ad libitum. A second group initially with weights of 240 to 272 grams was maintained on an ethanol-rich diet for five weeks (DeCarli and Lieber, 1967). During this period individual weight gains of 25 to 60 grams were observed. Both groups were killed by decapitation and their livers were removed.

Three Macaca mulattas and one M. fasicularis were killed under ketamine - nembutal anaesthesia and the livers were excised. The monkeys were maintained on a stock diet and weighed 3 to 4 kg. They were fasted overnight prior to sacrifice. They were killed primarily to obtain kidney and brain for other studies. Livers from four other M. mulattas maintained on a high cholesterol - sugar diet for another study and weighing 8 to 15 kg were also used (Table 9.4 experiments 5-8).

Incubations

Rat experiments. In each experiment two flasks containing liver slices and two control flasks were incubated. All flasks contained 30 ml of medium of identical electrolyte composition to that detailed on page 7.2. Sorbitol was present in each flask at a concentration of 0.1 mg per ml. Ethanol was at a concentration of 1 mg or 3 mg per ml in different experiments. The composition of the four flasks is shown in Fig. 9.2. Thus to flasks R and RC 2-3 uCi of (R)-[1-^{3}H] ethanol and 0.4-0.5 uCi of [1-^{14}C] ethanol was added.

Fig. 9.2. RADIO-CHEMICAL CONTENT OF INCUBATIONS WITH MEDIA CONTAINING LABELLED ETHANOL AND SORBITOL



The two other flasks, S and SC contained 3-4 uCi of $[2\text{-}^{3}H]$ sorbitol and 1-1.2 uCi of $[2\text{-}^{14}C]$ sorbitol. Paired flasks e.g. R and S therefore differed only in their radiochemical content. Alternate slices of the livers of two rats were distributed between flasks R and S. Between 3.6 and 5.2 grams of slices were added to each flask. Weights of slices in paired flasks differed by no more than 100 mg. No slices were added to RC and SC which served as controls.
The animals diets and medium ethanol concentrations in different experiments are shown in Tables 9.1-9.4.

Monkey experiments. A similar experimental design was adopted in the 8 experiments using monkey liver. The slices in each experiment were obtained from the liver of a single monkey. Ethanol concentration in these experiments was 3 mg/ml.

The flasks were gassed with 95% O_2/5% CO_2 for 10 minutes, stoppered and shaken at 37^{o} for 90 minutes at ethanol concentrations of 1 mg/ml and 2 hours at concentrations of 3 mg/ml. Incubation was terminated by the injection of 3 ml of 2N H_2SO_4. In this series of experiments carbon dioxide was not collected (c.f. chapter 7).

The acidified medium with slices from each flask was cooled, homogenised, centrifuged, neutralised, deproteiniated and recentrifuged as described in chapter 7. The resultant supernatant was made up to a constant volume of 70 ml with water. Acidified medium from control flasks was treated in a

similar manner. The concentration of ethanol was determined (Bonnichsen and Theorell, 1951) and the uptake from the medium calculated. No change in ethanol concentration was noted in the control flask as a result of incubation. The subsequent processing of the supernatant is outlined in Fig. 9.1.

Distillation and Determination of dpm in Ethanol and Water

In these experiments using the oxidiser carrier ethanol was added in excess to the supernatant prior to distillation (c.f. Chapter 7 and Fig. 7.1). Thus a 10 ml aliquot of each supernatant was made alkaline with 1N NaOH, 1 ml of 20% ethanol was added and the solution was distilled. For the rationale of the distillation procedure see Chapter 7 p. 7.3. From the first two ml of distillate the p-nitrobenzoate derivative of ethanol was prepared (Henstock, 1933). Weighed amounts of this ester were oxidised to water and carbon dioxide which were collected separately and assayed for radioactivity. The dpm in tritium and ^{14}C taken up from the medium were calculated from a knowledge of the weight of ester combusted and the quantity of ethanol added as carrier. No radioactivity was recovered in the nitrobenzoate derivative prepared from control flasks (i.e. without slices) containing labelled sorbitol. However in those flasks containing slices and radioactive sorbitol as tracer tritium but not ^{14}C was recovered from the p-nitrobenzoate derivative.

The seventh ml of distillate was combusted and the water and carbon dioxide was assayed for radioactivity. The formula on p. 7.5. was used to calculate the total dpm in tritiated water. In aliquots prepared from flasks containing labelled ethanol 15-20% of the tritium was present in ethanol.

The negligible amounts of ^{14}C in the distillate from flasks containing labelled sorbitol and of tritium in $^{3}H_2O$ in control incubations supports the validity of this method.

Determination of dpm present in Sorbitol

Carrier sorbitol was added to an aliquot of the supernatants from incubations employing labelled sorbitol (Fig. 9.2). The aliquot was then incubated with glucose oxidase in phosphate buffer pH 5.6 to convert glucose to gluconic acid. This was removed by passage through an Amberlite MB-3 column. The effluent was concentrated by evaporation and chromatographed on a Whatmann 3MM paper using butanol:acetic acid:water (Fink et al., 1963) which separated sorbitol from any remaining glucose. The sorbitol area, identified by guide spots, was eluted with water, concentrated in vacuo and dried over P_2O_5. Pyridine and acetic anhydride were added and after reaction at 80^{o} water was added and the sorbitol hexacetate crystals were collected. A weighed amount was combusted and the water and carbon dioxide were counted. Identical results were obtained when paper chromatography was omitted and this step was omitted in the last four experiments. Recrystallisation of the hexacetates did not change their specific activities thus confirming purity. Sorbitol hexacetates prepared from the supernatants of flasks containing labelled ethanol and liver slices showed no incorporation of label.

Determination of dpm in Glucose

Aliquots of the supernatants from the incubations using labelled sorbitol were taken and their glucose content was determined (Bergmeyer and Bernt, 1965). They were deionised

by passage through an Amberlite MB-3 column. The effluents were concentrated by evaporation and glucose was isolated by paper chromatography using butanol:acetic acid:water (Fink et al., 1963). The quantity of glucose present was measured and then carrier glucose was added. The first of three portions of this glucose solution was combusted and counted for tritium and ^{14}C. A second portion was incubated with glucose oxidase and deionised to remove the gluconic acid so formed. The effluent obtained after thorough washing of the column was concentrated by evaporation, then combusted and counted for tritium and ^{14}C. The decrease in radioactivity following incubation with glucose oxidase was taken to represent dpm present in glucose. There was very little radioactivity in the first portion and this was completely removed by incubation with glucose oxidase. This provides evidence for the purity of the glucose extracted from paper. Glucose in the third portion was converted to glucosazone (Vogel, 1956) with the removal of hydrogen from carbon 2 of the glucose. The osazone was weighed, combusted and counted for tritium and ^{14}C. The osazone contained the same quantity of tritium and ^{14}C on a molar basis as the glucose of the first portion. This proves there was a negligible amount of tritium at position 2 of glucose at the completion of incubation.

Determination of dpm in Lactate

Carrier lactate was added to 10 ml of the supernatant from each incubation. It was extracted, purified on a celite column and converted to its phenacyl derivative as described in chapter 7 p. 7.5. A weighed quantity of the phenacyl

derivative was combusted and assayed for tritium. The omission of celite purification gave identical results and this procedure was therefore omitted in the last few experiments. The specific activity of the phenacyl derivatives did not change on recrystallisation confirming purity.

RESULTS

The results of incubations with liver slices are shown in Tables 9.1 to 9.4. Using slices from livers of rats fed on stock chow four experiments were done at medium ethanol concentrations at 1 mg/ml (Table 9.1) and seven at 3 mg/ml (Table 9.2). Seven incubations were performed at 3 mg/ml with slices from rats maintained on a ethanol-rich diet (Table 9.3). Table 9.4 records the results of incubations at a medium ethanol concentration of 3 mg/ml with monkey liver slices.

With the exception of two experiments (numbers 4 and 5, Table 9.3) the uptake of ethanol was similar in each pair of flasks. The proportion of ethanol uptake was greater at 1 mg than at 3 mg/ml but not the quantity of ethanol utilised. Uptake of ^{14}C of $[2\text{-}^{14}C]$ sorbitol ranged from 51 to 70% and was not reduced at the higher ethanol concentration. In most incubations the uptake of tritium from labelled ethanol and sorbitol was less than that of ^{14}C from these substrates. With both substrates much more tritium was recovered in water than in lactate. In flasks containing labelled sorbitol 2.2 to 15.7% of the medium tritium was incorporated in ethanol at termination. The last column records estimates of the percent of ethanol metabolism

TABLE 9.1

THE METABOLISM OF (R)[1-^{3}H, 1-^{14}C] ETHANOL AND [2-^{3}H, 2-^{14}C] SORBITOL AT AN ETHANOL CONCENTRATION OF 1mg/ml BY LIVER SLICES OF RATS FED ON STOCK CHOW

Expt. No.	Labeled Substrate	Ethanol Upt.%	Uptake % of Medium		% of Medium ^{3}H to:			Estimated % ADH		
			^{14}C	^{3}H	Lactate	Water	Ethanol	Expr. 5	Expr. 6	Expr. 7
1	Ethanol	85.1	81.1	66.7	9.88	94.5		111.5	112.7	112.7
	Sorbitol	90.0	74.8	64.3	6.54	69.2	3.9			
2	Ethanol	51.0	43.0	31.7	5.74	56.0		95.3	94.9	94.3
	Sorbitol	60.1	60.4	46.7	4.67	43.3	5.9			
3	Ethanol	55.6	46.8	29.3	7.97	18.2		83.7	83.4	82.5
	Sorbitol	51.0	53.2	51.6	12.3	18.1	2.2			
4	Ethanol	81.9	78.4	67.8	8.47	65.0		97.0	96.9	96.9
	Sorbitol	80.1	69.1	71.7	4.98	36.1	4.8			
Mean ± S.E.M. R		68.4±8.8	62.3±10	48.9±11	8.0±0.9	58.4±16		96.9±5.7	97.0±6	96.6±6.2
Mean ± S.E.M. S		70.3±8.9	64.4±4.8	58.6±5.7	7.1±1.8	41.7±11	4.2±0.8			

TABLE 9.2

THE METABOLISM OF (R)[1-^{3}H, 1-^{14}C] ETHANOL AND [2-^{3}H, 2-^{14}C] SORBITOL AT AN ETHANOL CONCENTRATION OF 3mg/ml BY LIVER SLICES OF RATS FED ON STOCK CHOW

Expt. No.	Labeled Substrate	Ethanol Upt.%	Uptake % of Medium		% of Medium ^{3}H to :			Estimated % ADH		
			^{14}C	^{3}H	Lactate	Water	Ethanol	Expr. 5	Expr. 6	Expr. 7
1	Ethanol Sorbitol	44.0 35.9	41.5 73.4	39.2 64.4	2.57 3.18	33.4 40.3	11.4	98.5	98	97.6
2	Ethanol Sorbitol	39.0 31.3	31.6 75.4	35.2 62.2	1.99 3.7	24.1 37.0	9.1	88.9	87.5	86.3
3	Ethanol Sorbitol	27.5 20.8	15.6 66.3	10.7 55.1	1.99 4.22	18.6 36.4	10.6	94.9	93.3	93
4	Ethanol Sorbitol	29.2 24.5	9.4 63.8	7.5 56.4	2.82 4.39	24.5 32.6	15.7	91.0	87.8	86.7
5	Ethanol Sorbitol	30.4 40.7	29.0 72.2	11.2 57.3	3.53 5.1	18.8 21.4	6.8	90.8	89.7	88.9
6	Ethanol Sorbitol	35.2 35.0	26.2 66.3	18.3 52.8	1.96 5.06	15.8 25.8	8.5	77.9	75.2	72.8
7	Ethanol Sorbitol	24.9 26.9	27.0 68.0	15.4 58.2	1.67 4.44	10.7 26.0	6.4	96	95.5	95.3
Mean ± S.E.M. R		32.9±2.6	25.8±4	19.6±4.7	2.4±0.3	20.8±2.8		91.1±2.5	89.6±2.8	88.7±3.1
Mean ± S.E.M. S		30.7±2.7	69.3±1.6	58.1±1.5	4.3±0.3	31.4±2.7	9.7±1.2			

TABLE 9.3

THE METABOLISM OF (R) [1-^{3}H, 1-^{14}C] ETHANOL AND [2-^{3}H, 2-^{14}C] SORBITOL AT AN ETHANOL CONCENTRATION OF 3mg/ml BY LIVER SLICES OF RATS FED ON AN ETHANOL RICH DIET

Expt. No.	Labeled Substrate	Ethanol Upt.%	Uptake % of Medium		% of Medium ^{3}H to:			Estimated % ADH		
			^{14}C	^{3}H	Lactate	Water	Ethanol	Expr. 5	Expr. 6	Expr. 7
1	Ethanol	29.6	30.2	32.4	2.05	22.5		105.9	107	107
	Sorbitol	31.3	77.3	64.8	3.49	41.9	10.1			
2	Ethanol	23.6	20.1	14.9	1.45	19.3		107.9	109.6	109.6
	Sorbitol	24.1	60.9	47.1	2.16	32.2	7.7			
3	Ethanol	18.8	30.0	19.3	1.07	16.0		93.4	91.9	91.5
	Sorbitol	19.8	50.9	36.5	1.05	13.1	7.2			
4	Ethanol	11.1	17.5	9.7	0.86	15.7		92.0	90.8	90.4
	Sorbitol	23.0	63.6	49.9	1.36	20.5	6.9			
5	Ethanol	16.1	18.9	9.7	0.87	16.4		96.9	96.3	96
	Sorbitol	29.2	63.0	48.2	1.16	20.3	8.0			
Mean ± S.E.M. R		19.84±3.2	23.3±2.8	17.2±4.2	1.3±0.2	18±1.3		99.2±3.3	99.1±3.9	98.9±4
Mean ± S.E.M. S		25.5±2.1	63.1±4.2	49.3±4.5	1.8±0.5	25.6±5.1	8.0±0.6			

TABLE 9.4

THE METABOLISM OF (R)[1-^{3}H, 1-^{14}C] ETHANOL AND [2-^{3}H, 2-^{14}C] SORBITOL AT AN ETHANOL CONCENTRATION OF 3mg/ml BY LIVER SLICES OF MONKEYS FED ON STOCK CHOW* AND HIGH CHOLESTEROL† DIETS

Expt.No.	Labeled Substrate	Ethanol Upt.%	Uptake % of Medium		% of Medium ^{3}H to :			Estimated % ADH		
			^{14}C	^{3}H	Lactate	Water	Ethanol	Expr.5	Expr.6	Expr.7
1*	Ethanol	48.4	56.0	29.3	2.71	17.2				
	Sorbitol	48.1	49.0	38.4	1.61	9.2	5.9	97.4	96.9	96.4
2*	Ethanol	23.9	12.7	8.4	1.55	10.4				
	Sorbitol	24.1	36.8	22.2	1.84	6.4	4.3	78.9	75.1	73.6
3*	Ethanol	24.9	30.0	39.9	1.24	13.9				
	Sorbitol	23.2	39.7	26.4	1.38	9.4	4.8	81.3	78	76.9
4*	Ethanol	36.8	33.0	14.8	1.92	11.6				
	Sorbitol	36.5	68.2	50.3	2.7	13.0	10.6	93.8	92.3	91.1
5†	Ethanol	63.3	48.2	46.5	1.02	7.2				
	Sorbitol	64.2	63.0	51.8	1.29	7.5	4.4	97.0	96.7	96.2
6†	Ethanol	62.5	43.7	51.9	1.88	6.7				
	Sorbitol	64.2	62.3	47.3	1.9	6.5	8.2	99.4	99.3	99
7†	Ethanol	36.7	36.2	25.8	2.23	9.4				
	Sorbitol	40.1	36.8	33.8	2.49	9.5	3.8	97.1	96.8	96.7
8†	Ethanol	27.2	30.6	14.7	1.65	10.6				
	Sorbitol	33.1	55.5	44.4	1.73	7.4	11.0	92.3	90	88.5
Mean ± S.E.M. R		40.5±5.7	36.3±4.7	28.9±5.7	1.8±0.2	10.9±1.2				
Mean ± S.E.M. S		41.7±5.9	51.4±4.5	39.3±3.9	1.9±0.2	8.6±0.8	6.6±1.0	92.2±2.8	90.6±3.3	89.8±3.4

proceeding via alcohol dehydrogenase. The basis of these estimates will be discussed in the following section.

No more than 1.5% (range 0.2 to 1.5% in six incubations) of the tritium added as $[2-^{3}H]$ sorbitol was incorporated into glucose. These data ~~is~~ are not included in the table.

DISCUSSION

A medium ethanol concentration of 3 mg/ml in addition to 1 mg/ml was selected because of the higher Km reported for MEOS (Lieber and DeCarli, 1970). Livers from ethanol-fed rats were examined because of the reported higher MEOS activity resulting from this pre-treatment (see Chapter 1, p. 1.15). However in the present study the overall ethanol oxidation estimated from medium uptake is if anything lower in such rats (compare Table 9.2 with Table 9.3). Four of the eight monkeys were previously maintained on a high cholesterol-sugar diet (Table 9.4, experiments 5-8). It is not considered likely that this would exert any major influence on the oxidation of ethanol and the data from the eight experiments have been pooled. Monkey liver was used in view of the increased MEOS activity demonstrated in vitro in previous experiments (Chapter 6). Furthermore it was considered that data obtained in a primate might have more relevance to human biochemistry. The experiments were designed so that paired flasks differed only in the isotopic labelling of the two substrates. The similar uptakes of ethanol observed in paired flasks supports this comparability. Sorbitol was present at only 1/40 to 1/120 of the molar concentration of ethanol. This was chosen so that sorbitol metabolism would not influence the oxidation of ethanol via

redox changes. Steady state conditions were achieved in the present experiments by the selection of an appropriate medium volume, weight of slices and time of incubation. Thus significant quantities of substrates have been present throughout the incubations.

Sorbitol metabolism is assumed to proceed exclusively and irreversibly via sorbitol dehydrogenase to fructose (Morrison et al., 1972). Evidence for this irreversibility is the minimal incorporation of tritium into glucose in flasks containing [2-^{3}H] sorbitol. Even this small amount of tritium could have originated from labelled NADH. The lack of incorporation of tritium into sorbitol from labelled ethanol also provides evidence for the irreversible nature of sorbitol oxidation. The transfer of tritium to ethanol from [2-^{3}H] sorbitol is to be expected from the reversible nature of oxidation via alcohol dehydrogenase. This supports the presence of a common cytoplasmic pool of NADH. The influence of this incorporation of tritium in ethanol on the quantification of pathways is discussed below.

The data have been analysed according to the theoretical model outlined in chapter 3. The following formula is derived from the example given in Fig. 3.1 in which flask A contains labelled ethanol and flask B contains labelled sorbitol. It is assumed that during ethanol oxidation 1-(R) tritium is transferred either via alcohol dehydrogenase to NADH (X d.p.m.) or via MEOS/catalase to water (Y d.p.m.). Therefore the % metabolism proceeding via alcohol dehydrogenase is given by:

$$\frac{100.X.}{X + Y}$$ expression 1

All tritium dpm. transferred by MEOS/catalase appear directly in water. However only a fraction of the tritium in the NADH pool will be incorporated into lactate. This proportion may be determined from the fate of transferred tritium derived from labelled sorbitol in flask B. Thus X may be calculated as follows:

$$X = \frac{L(R) \times upt.(S)}{L(S)}$$ expression 2

In this L(R)* represents the ^{3}H dpm recovered in lactate from flask A, upt.(S) represents the ^{3}H dpm taken up from the medium in flask B and L(S) represents the ^{3}H dpm recovered in lactate in flask B. The content of tritiated water in the medium of flask A W(R) originates via MEOS/ catalase and also from NADH via mitochondrial oxidation. The amount of tritiated water in flask A derived from NADH is given by:

$$\frac{L(R) \times W(S)}{L(S)}$$ expression 3

and thus $$Y = W(R) - \frac{L(R) \times W(S)}{L(S)}$$ expression 4

in which W(S) represents the dpm in tritiated water in the medium of flask B at termination. Expressions 2 and 4 may be substituted into expression 1 to give expression 5:

% oxidation via ADH =

$$\frac{(\%L(R) \times \%upt(S) \div \%L(S)) \times 100}{(\%L(R) \times \%\ upt(S) \div \%L(S) + (\%W(R) - \frac{\%L(R) \times \%W(S)}{\%L(S)})}$$

*in this notation (R) refers to the flasks containing (R) $[1\text{-}^{3}H]$ ethanol and (S) to flasks containing $[2\text{-}^{3}H]$ sorbitol.

The dpm are now expressed for convenience as a percentage of the total dpm present in the medium at onset. Values calculated by this formula are given in Table 9.1 to 9.4.

The effect of the incorporation of tritium into ethanol in flasks containing labelled sorbitol on the quantification of pathways must now be considered. This incorporation will reduce the amount of tritium in the cytoplasmic NADH pool. Thus the uptake of tritium from medium sorbitol will not equal the dpm donated to a freely transferable NADH pool. This may be allowed for by subtracting the % ^{3}H dpm in ethanol at termination from %upt(S) in expression 5. This is referred to in Tables 9.1 to 9.4. as expression 6.
There is in addition a further problem to consider.
If MEOS/catalase exists it will oxidise a proportion of the (R)-$\left[1-^{3}H\right]$ ethanol formed from labelled sorbitol in flask B. This will result in the formation of further tritiated water. This incorporation will cause an additional overestimate of tritium donation to the NADH pool. It will also increase %W(S) by dpm that do not originate directly from this pool. The amount of tritium incorporated in $^{3}H_{2}O$ via MEOS in flask B, q. equals:

% dpm in EtoH x $(\alpha/1-\alpha)$ x proportion of oxidation via MEOS/CAT = q.

in which α represents the proportion of ethanol metabolised. Therefore allowing for both the incorporation of tritium in ethanol and for its metabolism in flask B via MEOS/catalase the following modifications of expression 1 are required:

$$X = \%L(R) \times \frac{(\%\ upt(S) - dpm\ in\ EtoH - q.)}{L(S)}$$

$$Y = \%W(R) - \frac{L(R) \times (W(S) - q.)}{L(S)}$$

This is expression 7. Estimates using this expression are given in Tables 9.1 - 9.4. In calculations using expression 7 the proportion of oxidation via MEOS/CAT derived from expression 6 has been used.

It is evident from Tables 9.1 to 9.4 that the modifications of expression 5 to allow for the transfer of tritium to ethanol from sorbitol (expression 6) and for its oxidation by MEOS/catalase (expression 7) produce only a small decrement in the estimated % oxidation via alcohol dehydrogenase. This is because of the small proportion of the tritium in ethanol and also the calculated minor contribution of MEOS/catalase to overall ethanol oxidation. The following discussion refers to the figures for % metabolism via alcohol dehydrogenase derived from expression 7. Statistical significance of differences of mean estimates from a theoretical value of 100% has been tested using the one-sample t test (Diem & Lentner, 1970). In experiments using rat liver slices with a medium ethanol concentration of 1 mg/ml (Table 9.1) a value of 96.6% $\pm$ 6.2% (mean $\pm$ S.E.M.) is obtained. A similar estimate of 98.9% $\pm$ 4% is obtained with rats pre-treated with ethanol (Table 9.3). These two estimates do not differ significantly from 100%. Individual experimental values in excess of 100% are observed in 3 instances (Table 9.1, experiment 1; Table 9.3, experiments 1 and 2). Such theoretically impossible values must represent

a random experimental error and these estimates have therefore been included in calculating the means. In rats, on a standard diet with a medium ethanol concentration of 3 mg/ml (Table 9.2) a statistically significant mean value of 88.7% ± 3.1% is obtained ($p<.01$). In monkey liver (Table 9.4) a similar estimate of 89.8% ± 3.4% ($p<.01$ ~~$p<.005$~~) is obtained. These 2 estimates indicate that in rats and monkeys not previously exposed to ethanol one tenth of ethanol oxidation proceeds via a pathway other than alcohol dehydrogenase at ethanol concentrations of 3 mg/ml. This contribution is not seen in rat liver at 1 mg/ml. This is explicable on the basis of the reported higher Km of MEOS (Leiber and DiCarli, 1970) or to an increase in peroxidatic activity of catalase at higher ethanol concentrations (Chapter 1, p. 1.10). The similar value of 10% in these two species is at variance with the findings reported with microsomal preparations in Chapter 6. This may reflect the attenuation of normal biochemistry in such artificial preparations. The absence of a MEOS/catalase contribution in rats pre-treated with ethanol is difficult to explain. In this group of animals however the uptake of ethanol from the medium was not greater than that observed with rats fed on a standard diet. Furthermore the failure of ethanol to induce its own metabolism in these experiments has been reported by other workers as discussed in Chapter 1, page 1.4. The present data from intact cells without the use of enzyme inhibitors suggest that reported MEOS activity in subcellular preparations is not entirely a preparation artefact. It is impossible to distinguish between oxidative activity due to

MEOS or catalase using the present technique. The data from Chance's laboratory that Compound I levels respond to exogenous alcohol in liver (Chapter 1, page 1.11) suggests that catalase may be responsible for at least a proportion of this activity. Leiber's estimate of 20 to 25% via MEOS in the non-induced animal (Leiber, 1972) is more than double that arrived at from the present data. This over-estimation may again be a reflection of the difficulty in drawing quantitative conclusions from disruptive subcellular preparations.

Rognstad (1974) has examined ethanol oxidation in isolated liver cells during gluconeogenesis. He has compared the incorporation of tritium from $[2\text{-}^{3}H]$ lactate and (R)-$[1\text{-}^{3}H]$ ethanol into glucose and water and concluded that 35% of ethanol oxidation proceeds via non-ADH pathways. However the experimental method of Rognstad casts doubt on the veracity of this estimation as follows:

1. The hepatocytes used by Rognstad were obtained from rats fasted for 24 hours to induce gluconeogenesis. This metabolic state associated with change in the redox state of liver (Guma et al., 1971) could influence the mode of ethanol oxidation in the hepatocyte. The unphysiologically high lactate concentrations used could also influence the mode of ethanol oxidation via changes in the intracellular redox state.

2. Substrate concentrations were as follows:
Labelled ethanol flasks: ethanol 60mM lactate 10mM.
Labelled lactate flasks: ethanol 20mM lactate 30mM.
These concentrations in "paired" flasks differ markedly.

Furthermore the incubation conditions would be expected to influence the fate of tritium transferred from (R)-[1-^{3}H] ethanol. An examination of Table 1 reveals that most of the [2-^{3}H] lactate was utilised (at 30mM) but that most of the labelled ethanol was not. Thus in flasks containing labelled ethanol as tracer significant depletion of lactate (at initial concentrations of 10mM) could occur. It follows that in those flasks containing (R)-[1-^{3}H] ethanol alcohol oxidation would be proceeding in conditions of reduced or absent gluconeogenic substrate viz., lactate for part of the incubation. This would favour incorporation of tritium into water rather than glucose and could give a fallacious over-estimate of non-ADH pathways.

3. Rognstad assayed tritium in medium water in flasks containing labelled lactate by counting medium after removal of lactate by deionisation. However as shown in the experiments described in this chapter tritium may be transferred to alcohol from the NADH pool in significant amounts due to the reversible nature of the alcohol dehydrogenase reaction. (R)-[1-^{3}H] ethanol so produced would give a falsely high estimate of incorporation of tritium into water and thereby result in an over-estimation of non-ADH pathways.

4. Rognstad assumes that all utilised tritium appears in glucose and water. Because of the incorporation of tritium into other compounds this also would result in an over-estimation of non-ADH pathways.

In all four series of experiments (Tables 9.1 to 9.4) the uptake of ^{14}C from medium ethanol and sorbitol exceeds

that of tritium. This enrichment of medium substrates with tritium indicates an isotopic discrimination versus protium in the oxidation of these two radio-chemicals. Such isotopic discrimination results from the presence of a primary kinetic isotope effect operating at a rate-limiting step (Rose, 1961). As discussed in Chapter 2 different workers have obtained values of KH/KD between unity and 1.6 with alcohol dehydrogenase *in vitro*. However, any isotopic discrimination involving the alcohol dehydrogenase pathway *in vivo* probably arises at the stage of disposal of tritiated cytoplasmic NADH i.e. at the rate limiting step in that pathway (Chapter 1, page 1.7). The present estimates assume that the non-ADH pathways do not have significantly different isotopic discriminations from that involving alcohol dehydrogenase. If isotopic discrimination was greater with these alternative oxidative mechanisms the present estimates of the contribution of alcohol dehydrogenase will be falsely high. On the other hand if they exhibited a lesser isotopic discrimination the present estimates of the non-ADH contribution will be excessive. Rognstad (1974) has shown that inhibition of alcohol dehydrogenase with pyrazole does not influence the observed isotopic discrimination in isolated hepatocytes metabolising ethanol. This suggests that the magnitude of isotopic discrimination is similar in the different pathways and that the present estimates are valid. However in the absence of any direct data on the relative isotopic discrimination in these different pathways the operation of such a source of error cannot be excluded.

POSSIBLE DEVELOPMENTS OF THE ISOTOPIC METHOD

The isotopic method described herein has proved suitable for examining the oxidation of ethanol in liver slices, from rat and monkey. Experiments could also conceivably be done using human liver obtained at operation. The method has the advantage of not requiring the use of metabolic inhibitors or homogenisation of tissues. The physiological nature of the preparation is supported by the large proportion of ethanol metabolised, the "physiological" concentration of medium acetaldehyde and the isotopic evidence for reversibility of ethanol oxidation. However a suspension of liver slices in artificial medium serves as an imperfect model for metabolism *in situ*. Ethanol and sorbitol are both metabolised mainly in the liver and adaptation of the method to the entire animal should be possible. This could be attempted initially in the rat and subsequently in the monkey and man. Tritiated sorbitol and ethanol can be infused under steady state conditions and the incorporation of tritium into lactate and water examined. This would involve the administration of these two radiochemicals on two separate occasions. A source of error would thus be introduced due to temporal variation. This could be very significant in conditions of changing metabolic state such as sudden abstinence after a period of high ethanol uptake. An alternative method would be to infuse concurrently one substrate labelled with tritium and the other labelled with deuterium. The fate of these two tracers could then be followed separately.

The method should be safe in man and no serious ethical problems are envisaged. Tritium emits only β-particles of low energy, is distributed throughout the body water and elimination is rapid. The estimated dose per experiment (0.1 mCi) is small. Initially experiments could be undertaken in volunteers to quantitate the pathways in normal man and determine the influence of factors such as age and sex. Studies could then be performed in alcoholics during and following excessive indulgence. Pathways could be quantified in the blood relativesof alcoholics to investigate possible genetic influences on metabolism. The effects of excessive intake of ethanol on normal volunteers could also be examined as could the effect of drugs known to influence the microsomal oxidation system. The biochemical basis of alcoholism and of the induction of ethanol metabolism in man is poorly understood because of inadequate methodology. It is the authors earnest hope that the present method will be applicable to the human subject and will throw some light on the biochemistry of man's oldest drug.

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THE OXIDATION OF ETHANOL BY MAMMALIAN LIVER

APPENDIX - INSERTION OF PUBLISHED PAPERS

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Stereospecificity of the Oxidation of Ethanol by Catalase*

(Received for publication, October 5, 1973)

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SUMMARY

(*R*)-[1-$^3H_1$]ethanol and (*S*)-[1-$^3H_1$]ethanol were oxidized to acetaldehyde using bovine hepatic catalase. The acetaldehyde formed from the *S* but not the (*R*)-[1-$^3H_1$]ethanol contained tritium. Thus the stereospecificity of catalase for alcohol is the same as that of alcohol dehydrogenase.

The primary action of catalase, the ability to decompose hydrogen peroxide with the evolution of oxygen, was originally described by Thénoud in 1911 (1). Keilin and Hartree (2) later showed that catalase was also capable of oxidizing a wide variety of compounds including ethanol in the presence of a hydrogen peroxide-generating system. The present study was undertaken to determine the stereospecificity of this catalatic oxidation of ethanol to acetaldehyde.

EXPERIMENTAL PROCEDURE

Materials

Acetaldehyde from Eastman Kodak was stored frozen in aqueous solution at −20°. Sodium (*R*,*S*)-[2-^{3}H]lactate (NET-025) was purchased from New England Nuclear. The following enzymes were purchased from Sigma: lactic dehydrogenase-L2625 from beef heart type III, yeast alcohol dehydrogenase-A7011, glucose oxidase-NG6125 type II and catalase-C100 from beef liver twice recrystallized. Nicotinamide adenine dinucleotide-15300 CNAA (NAD) was obtained from Boehringer, and the 3-acetyl pyridine analog of nicotinamide adenine dinucleotide-A5251 (AP-NAD) from Sigma. Dimetol reagent was made by dissolving dimethylcyclohexanedione (Eastman Kodak), 4 mg per ml, in acetate buffer, pH 4.2, 0.1 M. Counting was performed on a Nuclear Chicago 727 scintillation counter. Aquasol (New England Nuclear Corporation) was used as scintillant. All counts were converted to disintegrations per min by internal standardization.

Methods

Preparation of (R)-[1-$^3H_1$]Ethanol and (S)-[1-$^3H_1$]Ethanol—The methods described herein are similar in principle to those reported by Loewus *et al.* (3) for preparation of the deuterated derivatives. (*R*)-[1-$^3H_1$]Ethanol was prepared by the enzymatic reduction of acetaldehyde using sodium (*R*,*S*)-[2-^{3}H]lactate with lactic dehydrogenase and alcohol dehydrogenase. In a stoppered flask 1 mg of lactic dehydrogenase, 0.65 mg of alcohol dehydrogenase, 1.25 mg of NAD, 9.65 mg of lithium (*R*,*S*)-lactate, 7 mCi of sodium (*R*,*S*)-[2-^{3}H]lactate, and 32 mg of acetaldehyde were incubated at 37° in 9.65 ml of phosphate buffer, pH 7.4, 0.1 M. After 3 hours the reaction was terminated by the method of Somogyi (4).

TABLE I

Oxidation of enantiomers of [1-^{3}H]ethanol by yeast alcohol dehydrogenase with the 3-acetyl pyridine analog of nicotinamide adenine dinucleotide as coenzyme

Results of the oxidation of the tritiated ethanols are expressed as the specific activity dpm × 10^{-6} per mmole of the alcohol incubated with alcohol dehydrogenase and the specific activity of the acetaldehyde produced.

	Ethanol specific activity	Acetaldehyde specific activity	Ratio of specific activity acetaldehyde to specific activity ethanol
(*R*)-[1-$^3H_1$]ethanol	1.4	0.026	0.019
(*S*)-[1-$^3H_1$]ethanol	1.79	1.81	1.01

(*S*)-[1-$^3H_1$]Ethanol was prepared in two steps. In the first step, 9.6 mg of lithium (*R*,*S*)-lactate with 8 mCi of sodium (*R*,*S*)-[2-^{3}H]lactate was oxidatively decarboxylated to [1-^{3}H]acetaldehyde (5). This was collected in 5 ml of ice-cold water. In the second step, the aqueous [1-^{3}H]acetaldehyde was added to 10.7 ml phosphate buffer, pH 7.4, 0.2 M, containing 160 mg of lithium (*R*,*S*)-lactate, 1.25 mg of NAD, 0.65 mg of alcohol dehydrogenase, and 1 mg of lactic dehydrogenase. This was incubated in a stoppered Erlenmeyer flask at 37° for 3 hours when the reaction was terminated (4).

Both ethanols were collected in aqueous solution by distillation. Contaminating acetaldehyde was removed by combination with semicarbazone and the ethanols were redistilled under reduced pressure at 20°. The enantiomers were contaminated by less than 0.6% of tritiated acetaldehyde. When necessary, this contamination can be reduced to much less by repeated purification with semicarbazone.

Proof of Structure—The putative structure was tested by oxidation to acetaldehyde using alcohol dehydrogenase with AP-NAD as coenzyme[1] in pyrophosphate buffer, pH 9.0, 0.06 M. The acetaldehyde was distilled into dimetol reagent and precipitated overnight as dimetoacetaldehyde (5). This was washed with 500 ml of water in a Buchner funnel and dried at 60° under reduced pressure. The weighed precipitate was dissolved in Aquasol and counted. The specific activity of the acetaldehyde (Table I) approximated closely to that of the parent ethanol in the case of (*S*)-[$^3H_1$]ethanol. In contrast, when (*R*)-[1-$^3H_1$]ethanol was the substrate, only 1.85% of the tritium was retained in the acetaldehyde. This small fraction of the total tritium content may represent tritium in the *S* position and suggests the possibility that alcohol dehydrogenase does not show absolute stereospecificity. Each of the enantiomers was oxidized to acetic acid by chromic acid. The acetic acid from each contained less than 0.09% of the radioactivity of the corresponding ethanol, indicating that this ectopic tritium is not in the 2 position.

Catalatic Oxidation of Ethanol—The method was modified after Keilin and Hartree (2). In a stoppered 50-ml pear-shaped flask,

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[1] AP-NAD was preferred to NAD because of its more favorable equilibrium.

TABLE II

Catalatic oxidation of ethanol

Specific activities of the ethanols and the acetaldehydes formed from them are expressed as dpm $\times 10^{-6}$ per mmole. Corrections have been made for the contributions of contaminating tritiated acetaldehyde (not more than 0.6% of the added dpm) in the [1-$^3H_1$]ethanol preparations.

Experiment	^3H Position	Per cent ethanol oxidized	Ethanol specific activity	Acetaldehyde specific activity	Ratio of specific activity acetaldehyde to specific activity ethanol
1	*R*	95	1.37	0.028	0.020
	S	95	1.81	1.69	0.93
2	*R*	75	1.20	0.032	0.027
	S	84	1.31	1.32	1.01
3	*R*	77	1.20	0.030	0.025
	S	75	1.31	1.28	0.97

8 mg of ethanol with 0.1 μCi of tritium, 1 mg of catalase, 0.2 mg of glucose oxidase, and 60 mg of glucose were incubated at 25° in 12 ml of potassium phosphate buffer, pH 5.9, 0.2 M, for 15 hours. The flask was connected to a distillation apparatus and 5 ml were distilled into 8 ml of ice-cold dimetol reagent and the weighed precipitate was counted as described above. Identity of the dimetoacetaldehyde was confirmed by melting point determination (139°). Appropriate control experiments without catalase and with added carrier acetaldehyde were performed to allow correction for contaminating [1-^{3}H]acetaldehyde. A 0.1-ml aliquot was removed from the flask at termination, deproteinated, and assayed for ethanol (6) to determine the percentage of oxidation. In a separate experiment it was shown that the ethanol disappearance could be entirely accounted for by the accumulation of acetaldehyde assayed by the method of Gupta and Robinson (7).

RESULTS

The results of catalatic oxidation are shown in Table II. The specific activity of the resultant acetaldehyde on oxidation of the (*S*)-[1-$^3H_1$]ethanol approximated closely to that of the ethanol. With the (*R*)-[1-$^3H_1$]ethanol it was less than 3% of that of the ethanol. These results parallel those obtained with oxidation by alcohol dehydrogenase (Table I). To eliminate the possibility of contaminating alcohol dehydrogenase, the following experiment was performed. Catalase was added to a pyrophosphate buffer, pH 9.4, 0.075 M, containing ethanol, NAD, and semicarbazone and was incubated for 30 min at 37°. No change in optical density at 340 nm was observed after the addition of catalase. In a control flask to which alcohol dehydrogenase was added, a change in optical density of 0.67 was observed.

DISCUSSION

The catalatic oxidation experiments provide the first information on the stereospecificity of catalase. Table II indicated that the pro-*R*-hydrogen atom is selectively removed during catalatic oxidation. Fisher *et al.* (8) showed that alcohol dehydrogenase has an identical stereospecificity. This strict stereospecificity of catalase is remarkable in view of its broad spectrum for substrates including formaldehyde and nitrite (2). The results are clearly not an artifact of alcohol dehydrogenase contamination as shown by the experiment cited with NAD at pH 9.4. Furthermore, it is inconceivable that alcohol dehydrogenase could achieve almost complete oxidation of ethanol at pH 5.9 in the absence of a trapping agent for acetaldehyde. This stereospecificity of catalase may explain at least in part the high degree of orientation of the ethanol molecule with catalase that Sizer (9) predicted was necessary on thermodynamic grounds.

Note Added in Proof—After we had submitted this paper a communication (10) came to our attention that also reports that the stereospecificity of catalase for ethanol is the same as that of alcohol dehydrogenase.

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Table 1. *Hepatic peroxide and cytochrome P-450 concentration in rats after a single injection of phenobarbitone* (100 *mg/kg body wt.*)

Results are expressed as means ±S.D. *, indicates a value significantly different from that at time zero ($P<0.05$ by Student's t test).

Time after injection (h)	Cytochrome *P*-450 (nmol/g of liver)	Peroxide (μmol/g of liver)	No. of animals
0	23±4	0.09±0.04	8
3	23±4	0.15±0.06	9
6	22±3	0.50±0.13*	8
9	26±5	0.77±0.22*	6
12	31±3*	0.69±0.14*	4
24	57±7*	0.55±0.11*	3
48	58±9*	0.55±0.12*	6
72	43±7*	0.14±0.10	8

Male Wistar rats were used for the experiments; they were fed a standard laboratory diet (41B). Microsomal fractions were prepared, and cytochrome *P*-450 was measured, essentially by methods described elsewhere (Marshall & McLean, 1971). The peroxide content of liver homogenates was measured by an iodometric method (Wills, 1971).

The results discussed below indicate that peroxides do occur in the liver cell, and it has been shown (Hrycay & O'Brien, 1971) that cytochrome *P*-450 is a peroxidase. Table 1 shows hepatic peroxide and microsomal cytochrome *P*-450 concentrations in rats at various times after a single injection of phenobarbitone (100mg/kg, intraperitoneal), cytochrome *P*-450 concentrations are unchanged for 6h after giving the inducer; there may be some increase by 9h (though this is not statistically significant) and there is a significant increase after 12h. At 72h after the injection of inducer, the cytochrome *P*-450 concentration has started to decrease. In contrast, peroxide concentration in the liver increases rapidly between 3 and 6h so that at 6h there is a fivefold increase over the initial value. A high concentration is maintained while cytochrome *P*-450 concentration is increasing, but by 72h peroxide concentration has returned to the baseline. In rats given a 0.9% NaCl injection, there was no change in either cytochrome *P*-450 or peroxide concentrations at 12 or 24h from those at zero time.

In a group of chronically induced rats (given phenobarbitone in the drinking water at a concentration of 1mg/ml for 1 week), peroxide concentration was not significantly different from that seen in controls. However, once a new steady-state situation has been attained, the peroxide concentration required to maintain an increased concentration of cytochrome *P*-450 might only be slightly greater than that found in control situations, and the difference not detectable by the method used.

The value of enzyme kinetics in such complex systems as the microsomal fraction of a liver homogenate is dubious. However, we have observed enzyme kinetics that are consistent with competitive inhibition for the hydroxylation of aniline in the presence of peroxidized linoleic acid.

These results support the suggestion that the induction of hepatic microsomal cytochrome *P*-450 is mediated through an endogenous inducer, and that this inducer is a lipid peroxide or some related substance. Further work will be required to investigate the role of peroxides more fully. Paine & McLean (1974), working with a liver cell-culture system, suggested that the superoxide ion (O_2^-) may be a common intermediate in the induction of benzo(a)pyrene hydroxylase activity, another activity of the hepatic detoxicating enzyme.

Although the mechanism of enzyme induction in some bacterial systems is now well understood, the same is not true of mammalian enzyme induction. Yet this is a phenomenon of fundamental importance, enabling animals to adapt to changes in their

chemical environment and to different metabolic situations. Although the microsomal detoxicating enzyme is unique in its lack of specificity as far as both substrates and inducers are concerned, these observations may be of relevance to mammalian protein synthesis as a whole.

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Stereospecificity of the Microsomal Oxidation of Ethanol

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Fisher *et al.* (1953) have shown the oxidation of ethanol by alcohol dehydrogenase to be stereospecific, the 1-*R* hydrogen being selectively removed during oxidation to acetaldehyde. Corrall *et al.* (1974) have demonstrated that catalatic oxidation of ethanol exhibits an identical stereospecificity. The present study was undertaken to determine the stereospecificity of the microsomal ethanol-oxidizing system.

Ethanol was prepared, substituted with ^3H in the 1-*R* and 1-*S* positions, as previously described (Corrall *et al.*, 1974). These enantiomers plus [1-^{14}C]ethanol were incubated

Table 1. *Stereospecificity in the formation of acetaldehyde by the microsomal ethanol-oxidizing system*

The retention of ^3H by ethanol on oxidation to acetaldehyde is presented as the ratio of $^3H/^{14}C$ of the acetaldehyde produced to that of its parent ethanol. The pure microsomal suspension was prepared by a method similar to that of Leiber & De Carli (1970) and Isselbacher & Carter (1970), but involved two additional resuspension and centrifugation stages.

		$^3H/^{14}C$ in acetaldehyde/ $^3H/^{14}C$ in ethanol	
Animal	Method	*R*	*S*
Rat	Isselbacher & Carter (1970)	0.00	0.79
		0.10	0.64
	Leiber & De Carli (1970)	0.14	0.82
		0.13	1.00
	Pure	0.03	0.87
		0.16	0.81
	Catalase-free (Mezey *et al.*, 1973)	—	0.81
Monkey	Pure	0.03	0.94
		0.05	0.89

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in vitro with a variety of microsomal suspensions in the presence of appropriate coenzymes, buffer etc. The resultant acetaldehyde was precipitated as its dimedone derivative after addition of carrier acetaldehyde and the $^3H/^{14}C$ ratio determined (Table 1). ^3H is removed from the (*R*)-[1-^{3}H]ethanol but not from (*S*)-[1-^{3}H]ethanol indicating the stereospecificity to be the same as that of alcohol dehydrogenase and catalase.

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Studies on Obelin, the Calcium-Activated Luminescent Protein from the Hydroid, *Obelia geniculata*

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Calcium appears to play an important role in the control of a number of intracellular processes, including hormone action (Rasmussen *et al.*, 1972). At present there are no suitable techniques for studying the effects of hormones on the free intracellular concentration of calcium in small cells. Micro-injection of the luminescent protein aequorin has made it possible to study changes in the free intracellular calcium concentration in the giant axon of the squid (Baker *et al.*, 1971) and isolated barnacle muscle fibres (Ashley & Ridgeway, 1970). Aequorin is a protein of molecular weight about 30000, which can be extracted from the medusoid form of the hydrozoan *Aequorea forskålea* (Shimomura *et al.*, 1962). This protein luminesces in the presence of very low concentrations of calcium and appears to require no other substrates or cofactors. Unfortunately the jelly fish of aequorea rarely occur in any numbers off the coast of Britain. Studies on the hydroid form of the closely related *Obelia geniculata* have shown that this organism also contains a calcium-activated luminescent protein (Morin & Hastings, 1971). This hydroid occurs very commonly off the coast of Britain particularly in the South-West. The present report describes the extraction, partial purification and some of the properties of the luminescent protein, obelin, from this organism. These studies attempt to show that obelin is very similar to aequorin and that sufficient quantities can be obtained in this country to carry out physiological experiments.

Fronds of the brown seaweed *Laminaria* were collected by a skin diver in shallow water in Plymouth Sound during the months of August and September, 1973. The seaweed was thickly covered with *Obelia geniculata*. The hydroids were cut off, filtered, and the filtrate was assayed for luminescent activity. Luminescence was assayed by injecting a large excess of calcium (>5mM) into a solution of obelin at pH 8.9 placed in a specially constructed light-tight apparatus with a highly sensitive, low dark-current photomultiplier tube (Centronics P4232B). Very small quantities (<1 μl) of the extract were required for the assay, which was quantified by either measuring the total number of counts recorded on a scaler or by following the response on a chart recorder. The latter showed a peak within the response time of the recorder (approx. 100ms) followed by an exponential decay with a rate constant of $2.8\,s^{-1}$. This is similar to the value of $3.4\,s^{-1}$ reported by Morin & Hastings (1971). About 95–100% of the protein was consumed within 10s. Some examples of the total activity extracted are shown in Table 1.

Obelin reacts with very low concentrations of calcium. Once the protein has luminesced there is no known method of reactivating it. The stability of the crude extracts was dependent on (*a*) temperature (*b*) EDTA concentration (*c*) pH. The crude extract could

Table 1. *Total activity of some extracts*

The hydroids were extracted in 200mM-Tris+40mM-EDTA, pH7.0, at 3.5°C. A sma amount of the crude extract was diluted in 1ml of 200mM-Tris+40mM-EDTA, pH8.5 The total number of counts on the scaler were recorded during the 10s period after th injection of 1ml of 0.36M-$CaCl_2$.

Extract	Approximate extraction time (h)	Volume (ml)	Wet wt. of *Obelia* (g)	Total activity (counts)	Specific activity (counts/g)
IV	2h	500	31.8	2.4×10^{10}	7.6×10^{8}
XI	20h	1700	185.9	7.2×10^{11}	3.9×10^{9}
XII	20h	1300	113.4	6.9×10^{11}	6.1×10^{9}

be kept at 2–4°C at pH7.0 for a few days with little loss in activity. To prevent large losse in activity over longer time-periods the extracts were deep-frozen at −70°C or freeze dried and stored at −70°C. Only a small loss in activity was recorded after several month under both these conditions. It appeared that organisms themselves retained a conside able amount of luminescent activity after being stored for several months at −70°C.

Partial purification of the obelin was achieved by $(NH_4)_2SO_4$ precipitation. Abou 80–90% of the total protein, but less than 5% of the total luminescence activity wa precipitated by 60% satd. $(NH_4)_2SO_4$. The obelin was then precipitated with saturate $(NH_4)_2SO_4$, filtered on a glass-fibre filter and dissolved in 40mM-EDTA, pH7.0, th yield being 70% and the purification factor threefold. This partially pure extract wa stored at −70°C in EDTA alone or as an $(NH_4)_2SO_4$ suspension. The activity appeared t remain stable for several months.

Like aequorin obelin appeared to have a molecular weight of approximately 20000 o Sephadex G-75. Luminescence could be easily detected with concentrations of calciu as low as 100nM with calcium–EGTA [ethanedioxybis(ethylamine)tetra-acetic acid buffers (Portzehl *et al.*, 1964). Luminescence could also be stimulated by strontium a higher concentrations than those required for calcium. Barium and magnesium did no stimulate luminescence up to a concentration of 10mM, but magnesium inhibited th luminescence stimulated by calcium. Measurement of the steady-state luminescenc showed that the rate of light emission was proportional to the square of the free calciu concentration, as for aequorin (Baker *et al.*, 1971; Ashley & Ridgeway, 1970). In th presence of 1 or 10mM-Mg^{2+}, free calcium concentrations can easily be studied in th range 0.1–10μM, i.e. the physiological range inside cells. The minimum amount of tota activity required for steady-state measurements was 10^6–10^7 counts. This is considerabl less than has been injected into squid axons or barnacle muscle fibres, but it can be see from Table 1 that sufficient material should be available to carry out a large number o experiments.

Obelin appears to be very similar in its properties to aequorin. Extraction of obeli from *Obelia geniculata* should provide workers in this country with a readily availabl source of a calcium-activated luminescent protein for physiological experiments.

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SUBCELLULAR SITE OF ACETALDEHYDE OXIDATION IN RAT LIVER*

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Abstract—Slices of rat liver were incubated with (*R*)ethanol-1-^{3}H and (*S*)ethanol-1-^{3}H at a concentration of 1 mg/ml. During the course of the incubation, the ethanol in the flask containing the (*S*) isomer, but not the (*R*) isomer, was enriched with ^{3}H. For the same quantity of ^{3}H metabolized from the (*R*)ethanol-1-^{3}H as from the (*S*)ethanol-1-^{3}H, much less ^{3}H from the (*S*) than from the (*R*) isomer was incorporated into the lactate formed during the incubation. This indicates that the (*R*) hydrogen has a much greater access than the (*S*) hydrogen to the pool of NADH in the cytosol utilized in the reduction of pyruvate to lactate. It is concluded that the formation of NADH from acetaldehyde occurs under these conditions, primarily in a compartment other than the cytosol. It is presumed that this compartment is mitochondrial.

Until recently, acetaldehyde oxidation by the hepatocyte was generally believed to occur in the cytosol so that two equivalents of NADH were formed in the cytosol per mole of ethanol utilized. Reducing equivalents formed were assumed to be rapidly transported from the cytosol to the mitochondria via the malate–aspartate cycle to account for the rapid reduction of pyridine nucleotides occurring in the mitochondria of liver oxidizing ethanol [1]. More recently, the major portion of aldehyde dehydrogenase activity in rat liver has been localized to its mitochondria [2–4]. Most recently, Parrilla *et al.* [5] showed that inhibition of the extra mitochondrial reactions associated with the malate–aspartate cycle did not alter the reduction of mitochondrial pyridine nucleotides occurring in rat hepatocytes incubated with low concentrations of acetaldehyde but did to some extent with higher concentrations of acetaldehyde. They [5, 6] concluded that acetaldehyde at concentrations below 0·2 to 0·4 mM was oxidized predominantly in the mitochondria.

The present study was intended to localize the site of formation of NADH during acetaldehyde oxidation in the intact liver cell without recourse to inhibitors. The approach depends upon the stereospecificity of hydrogen removal in the oxidation of ethanol to acetaldehyde [7, 8]. The oxidation of 1 mole of (*R*)ethanol-1-^{3}H to acetaldehyde catalyzed by alcohol dehydrogenase will yield one equivalent of ^{3}H-labeled NADH, while the oxidation of (*S*)ethanol-1-^{3}H will yield one equivalent of unlabeled NADH. One equivalent of unlabeled NADH will be produced on oxidation of the unlabeled acetaldehyde formed from the (*R*)ethanol-1-^{3}H and one equivalent of ^{3}H-labeled NADH produced from the labeled acetaldehyde formed from (*S*)ethanol-1-^{3}H. Thus, for the same quantities of the (*R*) and (*S*) ethanols oxidized to acetate, the same quantities of labeled NADH will be formed. The ^{3}H should be on the same side of the pyridine ring of the nucleotide, whether from the (*R*) or (*S*) ethanol, since alcohol dehydrogenase and acetaldehyde dehydrogenase are A-type enzymes [9].

Therefore, if acetaldehyde oxidation, like ethanol oxidation, occurs in the cytosol, the cytosolic pool of NADH should be identically labeled with ^{3}H. The specific activity of the NADH pool formed from (*S*)ethanol-1-^{3}H relative to that from (*R*)ethanol-1-^{3}H should then reflect the extent of formation of NADH from acetaldehyde in the cytosol. These relative specific activities can be determined from the relative specific activities of the ^{3}H-labeled lactate formed during the metabolism of the (*R*) as compared to the (*S*)ethanol-1-^{3}H, since lactic dehydrogenase is localized to the cytosol and is also an A-type enzyme.

EXPERIMENTAL PROCEDURE

Materials. (*R*)ethanol-1-^{3}H, (*S*)ethanol-1-^{3}H and acetaldehyde-1-^{3}H were prepared and purified as previously described. Ethanol-1-^{14}C was purchased from New England Nuclear Corp., Boston, Mass., and Amersham/Searle Corp., Arlington Park, Ill., and also purified as previously described [8].

Animals. Sprague–Dawley white female rats weighing 260–300 g were fed *ad lib.* until the time of killing by decapitation.

Incubation. Five experiments were performed. In each experiment, six flasks with content were incubated. Each of the 500-ml Erlenmeyer flasks contained 30 ml medium. The medium [10] contained in m-moles/liter: K^+, 110; Mg^{2+}, 20; Ca^{2+}, 10; HCO_3^-, 40; Cl^-, 130; and glucose and ethanol, each at a concentration of 1 mg/ml. Ethanol-1-^{14}C (0·2 to 1·5 μCi) was added to all the flasks, (*R*)ethanol-1-^{3}H (1–5 μCi) to three of the flasks, and (*S*)ethanol-1-^{3}H (1–5 μCi) to the remaining three flasks. Flasks containing the (*R*) and (*S*) ethanols were paired. One pair, serving as controls, was incubated without addition of liver slices. Liver slices from two rats were randomly distributed into the second pair of flasks and liver slices from two other rats were distributed into

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the third pair. Between 3·6 and 4·4 g of slices was added to each flask. Paired flasks differed in weight of slices by no more than 0·1 g. The contents of the flasks were gassed with 95% O_2–5% CO_2 for 10 min and then stoppered and incubated at 37° with shaking for 90 min. To terminate incubation, 3 ml of 2 N H_2SO_4 was injected into each flask through a rubber inlet in the stopper. CO_2 evolved was collected in a vial which was suspended from each stopper and into which 3 ml of 1 N CO_2-free NaOH was introduced by injection through the inlet. In three of the experiments, 32 mg acetaldehyde in aqueous solution was injected through the inlet into each of the flasks after addition of the sulfuric acid.

Analyses. The sealed flasks were stored 12 hr to complete absorption of the $^{14}CO_2$ into the sodium hydroxide. The acidified medium with slices from each flask was then cooled in ice and homogenized. The homogenate in a closed bottle was centrifuged at 4°. The supernatant was neutralized, 1 ml of 0·3 N $Ba(OH)_2$ and 1 ml of 5% $Zn(SO)_4$ were added and the mixture was recentrifuged at 4°. The concentrations of ethanol in an aliquot of the supernatant and the initial medium were determined enzymatically [11]. From these determinations, the volume of medium incubated, and the volumes of the supernatant (corrected to the volume of the homogenate), the quantity of ethanol taken up during the incubation was determined. There was no disappearance of ethanol in flasks without added slices. A 10-ml aliquot of each supernatant was made alkaline with NaOH and distilled. The first ml of distillate was used to determine the amount of ^3H that remained in ethanol at the completion of incubation, the next 5 ml of distillate was discarded, and the next ml (the seventh ml) was used to determine incorporation of ^3H into water.

To the first ml of distillate and to an aliquot of the initial medium, carrier unlabeled ethanol was added and the *p*-nitrobenzoate derivative of ethanol was prepared from these aqueous solutions [12]. In the first three experiments, ethanol was then regenerated from each *p*-nitrobenzoate by refluxing it in 1 N NaOH and then distilling the resulting solution. An aliquot of the first portion of each of these distillates was added to scintillation fluid (Aquasol purchased from the New England Nuclear Corp.) and assayed for ^3H and ^{14}C. Internal standards were used in these and in all other assays of radioactivity. Ethanols assayed after formation of the *p*-nitrobenzoate from the distillate of the supernatants obtained in the incubations without slices had $^3H/^{14}C$ ratios identical to the ratios in the ethanols initially present in the incubation medium, providing evidence for the adequacy of the procedure.

The ratio of ^3H to ^{14}C in dis./min in the ethanol of each flask at the completion of incubation was calculated as follows. Aliquots of the initial solution were assayed for ^3H and ^{14}C and from these data the total ^3H and ^{14}C in dis./min added as ethanol was calculated. The ^3H present in the ethanol at the completion of incubation was calculated from the $^3H/^{14}C$ ratio in the ethanol at the completion of incubation, the ^{14}C in the ethanol at the beginning of incubation, and the fraction of ethanol that was not utilized as determined from the uptake of ethanol. For this calculation it is assumed that there is no isotope discrimination between ^{14}C and ^{12}C so that the percentage uptake of ethanol and of the ^{14}C of the ethanol-1-^{14}C is the same. In the last two experiments, the *p*-nitrobenzoates were oxidized to $^{14}CO_2$ and ^3H_2O in an oxidizer (model 306, Packard Instrument Co., Downers Grove, Ill.). Uptake of ^{14}C and ^3H could then be calculated directly from the aliquot taken, the quantity of nitrobenzoate oxidized, and its activity. The uptake of ^{14}C of ethanol-1-^{14}C was in good agreement with the uptake of ethanol determined enzymatically [11].

An aliquot of the seventh ml of the distillate was assayed for ^3H and ^{14}C content. All ^{14}C in this distillate was assumed to be in ethanol, and all ^3H other than that in ethanol was assumed to be in water. The ^3H in ethanol was estimated from the $^3H/^{14}C$ ratio in ethanol in the distillate, as just detailed, and the ^{14}C in the distillate. In the seventh ml of distillate, about 25 per cent of the total ^3H was thus found to be due to ethanol with the remainder ascribed to ^3H_2O. Negligible ^3H was found in ^3H_2O in the incubations without slices. When ^3H_2O was distilled in a control experiment, the specific activity of the initial and final distillate was the same. The total incorporation of ^3H into water has, therefore, been calculated from the ^3H in the aliquot of the distillate and the total volume of the supernatant.

To another aliquot of each supernatant, carrier lactate was added. The aliquot was acidified with H_2SO_4 and the lactate extracted with ether. The lactate in the ether was isolated as its sodium salt and purified on a Celite column [13]. Lactate from the column was converted to its phenacyl derivative [14], which was assayed for ^3H and ^{14}C. The incorporation of ^3H and ^{14}C into lactate was calculated from this ratio, the weight of the phenacyl lactate assayed, the quantity of lactate added as carrier, and the volume of the aliquot of the supernatant. Again in the last two experiments, the phenacyl lactates were oxidized and the $^{14}CO_2$ and ^3H_2O counted.

In the three experiments in which carrier acetaldehyde was added, an aliquot of the supernatant from each flask was distilled *in vacuo* at room temperature into dimedone reagent [8] contained in a glass-jointed tube immersed in ice water. After the collection of a few ml of distillate, the tube was stoppered and warmed to complete the formation of acetaldimedone, which was collected and assayed for ^{14}C and ^3H. The quantities of ^3H and ^{14}C in acetaldehyde in the incubation medium plus slices at the completion of incubation were calculated from the 32 mg acetaldehyde added as carrier and the weight of acetaldimedone assayed for radioactivity. Negligible ^3H and ^{14}C radioactivity was in the acetaldimedone isolated from the incubations without slices, except in one experiment where there was a quantity of ^{14}C, despite purification of the ethanol-1-^{14}C used. The ethanol-1-^{14}C was a new batch which, when purchased, had several per cent of its activity precipitated with dimedone reagent. The quantity of acetaldehyde present in the slices and incubation medium of each flask at the completion of incubation for the two experiments where there was no contamination was calculated from the total ^{14}C in acetaldehyde at the completion of incubation and the molar specific acti-

vity of the acetaldehyde-1-^{14}C formed during the incubation, assumed to be the same as that of the ethanol-1-^{14}C incubated.

A control experiment showed that no significant quantity of acetaldehyde was lost during the course of the incubation. Acetaldehyde-1-^{3}H (5 mg) was injected into medium with slices immediately after acidification and without incubation. It was then incubated for 90 min, carrier acetaldehyde (32 mg) was added and the above procedure was followed. Twenty-six mg acetaldimedone was recovered. Its specific activity was such that the theoretical yield of acetaldimedone from 37 mg acetaldehyde (257 mg) would have contained 97 per cent of the dis./min added to the flask.

The $^{14}CO_2$ absorbed into the sodium hydroxide was precipitated as $Ba^{14}CO_3$. The $Ba^{14}CO_3$ was weighed and then treated with H_2SO_4 and the $^{14}CO_2$ evolved collected in ethylenediamine in methylcellusolve [15] and assayed for ^{14}C. Incorporation into $^{14}CO_2$ was calculated from the specific activity of the $^{14}CO_2$ and the quantity of CO_2 estimated to be present in the medium and gas phase of each flask.

RESULTS

Results of the five experiments are presented in Table 1. Uptake of ethanol, measured enzymatically, was about 50 per cent of the added ethanol with very similar uptakes in the paired incubations. The per cent uptake of ^{3}H from (*R*)ethanol-1-^{3}H was similar to the per cent uptake of ethanol determined enzymatically, i.e. the $^{3}H/^{14}C$ ratio in the ethanol remaining at the completion of incubation was similar to, although somewhat higher than, the ratio in the ethanol at the beginning of the incubation. In contrast, the uptake of ^{3}H from (*S*)ethanol-1-^{3}H was only about half of the uptake of ethanol, i.e. enrichment of ^{3}H in ethanol occurred, so that the $^{3}H/^{14}C$ ratio in ethanol at the end of incubation was higher than that in ethanol at the beginning of incubation.

In support of a similar metabolism of ethanol-1-^{14}C in the flasks containing the (*S*) and (*R*) isomers are the similar yields of ^{14}C in $^{14}CO_2$. About four times as much of the uptake of ^{3}H from (*R*)ethanol-1-^{3}H as from (*S*)ethanol-1-^{3}H was recovered in lactate. However, of the uptakes of ^{3}H, a similar amount, about 60 per cent, was recovered in water from both isomers. The per cent of the ^{3}H uptake recovered in acetaldehyde was very small with the (*R*)ethanol-1-^{3}H. It was more with the (*S*)ethanol-1-^{3}H, but of the uptake of ^{3}H it was still a small percentage. In Table 2 are recorded the $^{3}H/^{14}C$ ratios in ethanol and acetaldehyde for the two experiments with the (*S*) isomer. The ratios in ethanol, as already noted, were higher at the completion than initiation of incubation. The ratios in acetaldehyde were the same as or greater than those in the ethanol remaining in the medium at the completion of incubation. Acetaldehyde in the reaction mixture at the completion of incubation in these experiments contained 0·1 to 0·4 per cent of the added ^{14}C; assuming that the specific activity of the acetaldehyde had the same molar specific activity as that of the ethanol-1-^{14}C, this calculates to a total acetaldehyde content of 30–120 μg.

Table 2. $^{3}H/^{14}C$ Ratio in ethanol at the beginning and completion and in acetaldehyde at the completion of incubation with (*S*)ethanol-1-^{3}H and ethanol-1-^{14}C

Expt.	Ethanol Initial	Ethanol Final	Acetaldehyde Final
1	2·53	3·27	3·28
	2·53	3·37	3·62
2	2·14	3·30	3·44
	2·14	2·80	2·81

DISCUSSION

Our major conclusion is that the (*S*) hydrogen of ethanol has much less access to the NADH pool used in pyruvate reduction than does the (*R*) hydrogen, when the ethanol is metabolized by rat liver slices at concentrations between 1·0 and about 0·5 mg/ml, the initial and final concentrations in the medium. This is concluded from the fact that for the same quantity of ^{3}H in ethanol-1-^{3}H utilized, much less ^{3}H from the (*S*) ^{3}H-labeled than from the (*R*) ^{3}H-labeled hydrogen is incorporated into lactate. Since lactate dehydrogenase is well documented to be in the cytosol, the specific activity of lactate must reflect the specific activity of this NADH pool.

Several explanations for the above observation are possible. Acetaldehyde could be oxidized by a process not involving NADH formation or by a dehydrogenase of the B-type. There are several aldehyde dehydrogenases in liver, including one with NADPH as a cofactor [3, 4, 16], and xanthine oxidase has, for example, been reported to catalyze the oxidation of acetaldehyde [17]. However, specificity, affinity and activity data would indicate that it is the NADH-dependent aldehyde dehydrogenases that are functionally important and the acetaldehyde dehydrogenase from bovine liver that has been examined has proven to be of the A-type [18].

The decreased incorporation of ^{3}H into lactate from the (*S*) compared to the (*R*) ethanol cannot be explained by an isotopic effect, since only a small amount of ^{3}H from the (*S*) ethanol that was utilized was recovered in acetaldehyde, while large, similar percentages of ^{3}H were recovered in water from the (*S*) and the (*R*) ethanol. Conceivably, separate NADH pools in the cytoplasm could exist, one having greater access to the hydrogen removed from ethanol than acetaldehyde, but there is a considerable amount of

Table 1. Metabolism by rat liver slice of (*R*)ethanol-1-^{3}H and (*S*)ethanol-1-^{3}H each in the presence of ethanol-1-^{14}C*

Ethanol isomer	% Ethanol uptake	% ^{3}H uptake	% ^{14}C uptake to CO_2	Lactate	% ^{3}H uptake to Water	Acetaldehyde
R	52·9 ± 2·2	44·2 ± 3·6	11·4 ± 2·0	11·7 ± 2·7	59·4 ± 4·5	0·07 ± 0·01
S	50·8 ± 2·3	27·4 ± 1·9	9·5 ± 1·6	2·7 ± 0·2	62·0 ± 3·3	1·6 ± 0·6

* Mean ± S. E.

data to support the presence of a single pool of NADH in the cytosol [19].

The most likely explanation is that the conversion of ethanol to acetaldehyde occurs in the cytosol, but the acetaldehyde oxidation occurs primarily in another compartment, presumably the mitochondrial compartment. The data indicate that under the conditions of our study at most one-quarter of the oxidation of acetaldehyde occurred in the cytosol.

In this discussion we have assumed ethanol oxidation to be catalyzed solely by alcohol dehydrogenase. There is evidence that catalase and a microsomal oxidizing enzyme system may contribute, at least to a degree, to the conversion of ethanol to acetaldehyde. In these oxidations the (*R*) hydrogen of ethanol is removed, but NADH is not formed [8, 20]. To the extent that these reactions occur rather than oxidation via alcohol dehydrogenase, the extent of the compartmentation determined with the (*R*) and (*S*) isomers would be underestimated.

As noted, the data of Parrilla *et al.* [5] and Lindros *et al.* [6] indicate that the oxidation of acetaldehyde occurs almost entirely in the mitochondrial compartment at aldehyde concentrations below 0·2 and 0·4 mM, concentrations normally encountered in ethanol metabolism. The 30–120 μg acetaldehyde estimated to be in the medium plus liver slices at the termination of incubation, even if present solely in the approximately 3 ml water in the slices, would give an acetaldehyde concentration of 0·2 to 0·8 mM.

The enrichment of ethanol with ^{3}H relative to ^{14}C during the incubation with the (*S*) isomer presumably reflects a primary isotopic effect during the dehydrogenation of acetaldehyde-1-^{3}H, since, as shown in Table 2, the acetaldehyde is enriched with ^{3}H relative to ^{14}C. Since the dehydrogenation of ethanol to form acetaldehyde, catalyzed by alcohol dehydrogenase, is readily reversible, resynthesis of ethanol from acetaldehyde would explain the enrichment of ^{3}H in the ethanol. We (P. Havre and B. Landau, unpublished observations) have performed experiments identical to those described in this study but with ethanol unlabeled and sorbitol-2-^{3}H added to the medium at a concentration of 0·1 mg/ml. Between 10 and 20 per cent of the ^{3}H taken up was recovered in ethanol, supporting the reversibility of the alcohol dehydrogenase-catalyzed dehydrogenation under the conditions employed.

Rognstad and Clark [21], using a theoretical approach similar to ours, compared the specific yield of ^{3}H in water and glucose formed by liver cells from (*R*) and (*S*)ethanol-1-^{3}H, with the specific yields from substrates oxidized by dehydrogenases with known cytoplasmic and mitochondrial locations. They also concluded that ethanol is oxidized predominantly in the mitochondria. Lactate was present in their incubation media at a concentration of 0·72 mg/ml (in one experiment pyruvate was substituted at a concentration of 1·32 mg/ml), and the (*R*) and (*S*) ethanols at the beginning of the 1-hr incubations were at concentrations between 0·001 and 0·01 mg/ml.

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Stereospecificity of the microsomal ethanol-oxidizing system*

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The microsomal ethanol-oxidizing system (MEOS) has been interpreted by Lieber and DeCarli [1] to be a unique system contributing significantly to ethanol metabolism *in vivo*. A number of investigators have considered the possibility that MEOS activity is due to catalase or catalase combined with alcohol dehydrogenase activity [2–4], but a number of recent studies support an MEOS function independent of catalase [5–8]. Teschke *et al.* [5, 9] and Mezey *et al.* [10] reported the isolation of a microsomal preparation free of or containing only small amounts of catalase and capable of converting ethanol to acetaldehyde. Thurman and Scholz [3] isolated in a similar manner a catalase-free microsomal fraction, but found it devoid of MEOS activity. Barakat *et al.* [11] examined ethanol oxidation by components of rat liver microsomes and found that highly purified cytochrome P-450 did not oxidize ethanol appreciably.

Alcohol dehydrogenase shows stereospecificity, oxidizing the *R* hydrogen of ethanol in the formation of acetaldehyde [12]. Corrall *et al.* [13] reported that catalase shows the same stereospecificity. This is a report of a study to determine if, in the oxidation of ethanol by MEOS, the *R* or *S* hydrogen is retained in the acetaldehyde formed.

(*R*)-[1-$^3H_1$]ethanol and (*S*)-[1-$^3H_1$]ethanol were prepared and [2-^{14}C]ethanol was purchased from New England Nuclear Corp., Boston, Mass. Initially, media as described by Isselbacher and Carter [14] and Lieber and DeCarli [15] were employed. Isselbacher and Carter incubated ethanol with microsomal material in the presence of NADPH, magnesium chloride, and a sodium phosphate buffer at pH 7·4, while Lieber and DeCarli employed a potassium phosphate buffer at pH 7·4 containing magnesium chloride, an NADPH generating system consisting of NADP, sodium isocitrate and isocitrate dehydrogenase, and in the presence of nicotinamide. All later incubations were with a potassium phosphate buffer (80 mM) at pH 7·4 with 0·3 mM NADPH and 5 mM magnesium chloride.

Microsomes incubated in the medium of Isselbacher and Carter [14] were prepared from a 20% liver homogenate in 0·25 M sucrose made from livers of female Sprague–Dawley rats killed by decapitation. The microsomal fraction collected after centrifugation at 105,000 *g* was resuspended in 0·15 M KCl [14, 16]. Using the medium of Lieber and DeCarli [15] microsomes were prepared by homogenization in 0·15 M KCl and, after centrifugation at 9000 *g* for 30 min, the crude microsomes were collected by centrifugation at 105,000 *g* and resuspended in KCl and incubated, or further purification was attempted by washing. For the latter, the KCl suspension was centrifuged again at 105,000 *g*. The resulting pellet was resuspended in KCl, followed by a third centrifugation at 105,000 *g* and then resuspension of the resulting pellet in KCl for incubation. Protein in the final microsomal suspension was determined using biuret [17].

Livers were obtained from three monkeys. The first, a male, had been fasted 72 hr and his liver was removed under phencyclidine anesthesia. The other two were females which had been fasted overnight and were under barbiturate anesthesia. The first monkey, 1 yr before sacrifice, was under hyperthermia for 48 hr for another study. The second monkey was being sacrificed after accidental hemorrhage in preparation for another study; the third monkey had been oophorectomized and the liver was removed at the time its brain was being isolated and placed under perfusion for another study. It is assumed that these circumstances do not alter the stereospecificity of ethanol oxidation, but could alter the quantity of ethanol utilized. The use of these monkeys was preferred to killing of monkeys for the present study. The livers were collected in cold isotonic saline and homogenized within 40 min of removal.

To each of two flasks were added the potassium phosphate buffer, magnesium chloride and NADPH to give the final concentrations noted, and the microsomal suspension to give a final protein concentration of 3 mg/ml. The flasks were stoppered with inlets in the stoppers to permit subsequent injections into the media. The gas phase was air, except that oxygen was used when the conditions of Isselbacher and Carter [14, 18] were employed. Flasks with contents were incubated for 10 min at 37°, and then labeled ethanol was injected. To each flask, 0·4 to 1·2 μCi of [2-^{14}C]ethanol was added and 1–3 μCi of (*R*)-[1-$^3H_1$]ethanol was added to one flask and 1–3 μCi of (*S*)-[1-$^3H_1$]ethanol to the other flask. The ethanol was added to give a final concentration in the 40-ml volume of 2·3 mg/ml. Incubation was for 20 min. Two additional flasks serving as controls were incubated identically, except that that the microsomal suspension was boiled before addition or KCl was substituted for the suspension.

Incubation was terminated by the addition of equal volumes 0·3 N $Ba(OH)_2$ and 5% $ZnSO_4$ to the flasks and then 4·8 mg of unlabeled acetaldehyde in water was injected. The content of each flask was cooled and centrifuged. The supernatant was distilled and the initial distillate was collected in dimetol reagent. The dimetoacetaldehyde that precipitated was collected, washed, dried and weighed [12].

Catalase-free MEOS was prepared essentially as described by Mezey *et al.* [10], except that female Sprague–Dawley rats [9] weighing 200–300 g were used. After isolation of the microsomal pellet and its solubilization, an ammonium sulphate precipitate was dialyzed. The dialyzed protein was passed through a Sephadex-G-25 column and was then placed on a DEAE cellulose column and eluted with KCl [10]. Catalase activity appeared at the beginning

* A preliminary account of these studies has appeared (*Biochem. Soc. Transactions* **2,** 994 1974).

of elution and a cytochrome P-450 peak followed which was devoid of catalase activity [3, 9, 10]. The tube fractions containing the P-450 peak were combined and ammonium sulfate was added to 50% saturation. The resulting precipitate was collected and dissolved in potassium phosphate buffer and incubated with NADPH, magnesium chloride and the labeled ethanols as just described, except that the total volume of the reaction mixture was 5 ml and protein was at 13 mg/ml. Catalase activity was determined by the method of Bergmeyer [19]; cytochrome P-450 was measured by the method of Omura and Sato [20].

Radioactivity in the acetaldehyde was assayed after dissolving dimetoacetaldehyde in a scintillant (Aquasol from New England Nuclear Corp., Boston, Mass.). All cpm were brought to dis./min by internal standardization. The dis./min actually incorporated into acetaldehyde were calculated from the 16–19 mg dimetoacetaldehyde collected and the 33·4 mg dimetoacetaldehyde to be expected from 4·8 mg of acetaldehyde, if there were no losses in isolation. Samples of the media incubated were assayed to obtain the total dis./min of ^{14}C and ^{3}H incubated.

^{14}C and ^{3}H present in the dimetoacetaldehyde isolated in the control incubations were due to contamination of the labeled ethanols with labeled acetaldehyde as evidenced by similar recoveries whether the microsomal suspension was boiled or 0·15 M KCl was substituted for the microsomal suspension and whether incubation was terminated at the beginning of incubation or after 20 min. By repeated distillations of the ethanols from semicarbazide before use [13], acetaldehyde contamination was reduced to less than 0·01 per cent of the dis./min added in the ethanols; the radioactivities in acetaldehydes in the control incubations were 1/10th or less of the activities in the acetaldehydes formed in the flasks incubated with the unboiled microsomal preparations. The activities in the acetaldehyde in the control flasks were subtracted from the corresponding activities in the acetaldehydes formed by MEOS. By incubating catalase-free MEOS at a protein concentration of 13 mg/ml, contaminating labeled acetaldehyde was also a minor quantity compared to the quantity of labeled acetaldehyde formed, despite the lower MEOS specific activities in these preparations.

While it is unlikely that a difference in stereospecificity would be observed consequent to the very small differences in conditions selected by Isselbacher and Carter [14, 18] compared to those of Lieber and DeCarli [15], stereospecificity was examined under both sets of conditions. The results are recorded in Table 1. The ratio is given of ^{3}H to ^{14}C in the acetaldehyde formed by the MEOS to the ratio of ^{3}H to ^{14}C in the ethanol incubated. There was 14 per cent or less retention of the *R* hydrogen, while the retention of the *S* hydrogen ranged from 64 to 100 per cent under these conditions. Using the modified incubation medium as described in Methods in all subsequent experiments, results were similar, with little or no tritium in acetaldehyde formed from the (*R*)-[1-$^{3}H_1$]ethanol, while 80 per cent or more of the tritium in the (*S*)-[1-$^{3}H_1$]ethanol oxidixed was retained.

The small retention of the (*R*)-hydrogen and incomplete retention of the (*S*)-hydrogen may indicate oxidation by more than one reaction. With the monkey preparations, the ratio with the (*R*)-hydrogen appears somewhat closer to 0; with the (*S*)-hydrogen it is closer to 1·0. Secondary isotopic effects could be responsible for the failure to obtain theoretically complete retention of the (*S*)-hydrogen. The approach to the theoretical ratio of 1·0 using crude monkey preparations may be due to the higher percentage of conversion of ethanol in these incubations, so that an isotopic effect would be obscured. Rognstad [8] has considered the contribution of catalase to ethanol oxidation, assuming it is the (*R*) and not the (*S*) atom which is responsible for isotopic discrimination. Gang *et al.* [21] reported a single experiment showing that racemically labeled [1-$^{3}H_1$]ethanol, on incubation with rat MEOS, yields acetaldehyde with much less than half of the specific activity of the [1-$^{3}H_1$]ethanol. This would suggest considerable isotopic discrimination for the (*S*)-hydrogen. The quantity of ethanol oxidized was not reported. The (*R*)-hydrogen from (*R*)-[1-$^{3}H_1$]ethanol was removed in accordance with the present report.

The stereospecificity of MEOS, the removal of the *R*-hydrogen in acetaldehyde formation, is the same stereospecificity that has been observed for alcohol dehydrogenase [12] and catalase [13]. However, we observed acetaldehyde formation with the same stereospecificity in an MEOS preparation [10] in which catalase and alcohol dehydrogenase have not been detected, although admit-

Table 1. Oxidation of (*R*)-[1-$^{3}H_1$]ethanol and (*S*)-[1-$^{3}H_1$]ethanol in the presence of [2-^{14}C]ethanol by microsomal preparation from rat and monkey

Animal	Microsomal preparation	Expt. No.	$^{3}H/^{14}C$ in CH_3CHO / $^{3}H/^{14}C$ in C_2H_5OH (*R*)-1-^{3}H	(*S*)-1-^{3}H	C_2H_5OH oxidized (nmoles/min/mg protein)
Rat	Crude	1*	0·00	0·79	2·1
		2*	0·10	0·64	1·4
		3†	0·14	0·82	3·5
		4†	0·13	1·00	2·8
		5	0·11	0·86	1·4
	Washed	1	0·03	0·87	0·7
		2	0·16	0·81	1·4
	Catalase-free	1	‡	0·81	0·6
		2	0·07	‡	0·4
Monkey	Crude	1	0·07	0·98	453
		2	0·05	1·03	238
		3	0·03	1·13	151
	Washed	1	0·02	0·95	73·8
		2	0·02	0·88	8·2
		3	0·03	0·91	12·5
	Catalase-free	1	0·02	0·80	1·5

* Incubation medium as described [14].
† Incubation medium as described [15].
‡ Incubations not done.

tedly with less formation of acetaldehyde than in the less purified microsomal preparations. A decrease in activity would be expected with solubilization of the microsomes.

The amount of ethanol oxidized to acetaldehyde, in nmoles per min of incubation per mg of microsomal protein, has been calculated from the specific activity of the [2-^{14}C]ethanol and the quantity of ^{14}C incorporated into the acetaldehyde. The quantity oxidized in the flask containing (*R*)-[1-$^{3}H_1$]ethanol was, as expected, essentially the same (within 10 per cent) as that in the flask with (*S*)-[1-$^{3}H_1$]ethanol in each experiment, and the average of these two values is recorded in the last column of Table 1. The quantity was greater with the monkey preparations. Little change in acetaldehyde formation as a result of the repeat washings of the rat microsomal preparation is evident, but the specific activity in nmoles of ethanol oxidized to acetaldehyde per mg of protein was least with the catalase-free preparation.

The acetaldehyde formed is less than the 14·7 nmoles/min/mg of protein reported by Lieber and DeCarli [22] for a 10-min incubation period. We have assumed linear formation of acetaldehyde over the 20-min period of incubation [15]. The greater rate of formation of acetaldehyde in the monkey could suggest that in the primate MEOS is more active. However, Mannering *et al.* [23] reported monkey liver has a larger quantity of catalase than rat liver, but is less active peroxidatively. MEOS prepared from human liver obtained by surgical biopsy is reported to have somewhat less ethanol-oxidizing activity than MEOS prepared from rat liver [15]. The amount of ethanol oxidized in the incubation with MEOS from monkey liver exceeds by many fold the amount of NADPH added. Vatsis and Schulman [24], making a similar observation, postulated the existence of a constituent in microsomes that regenerates NADPH during ethanol oxidation.

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UANTITATION OF PATHWAYS OF ETHANOL METABOLISM IN RAT AND MONKEY LIVER.

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Liver slices were incubated in paired flasks with ethanol (1 and 3 ng/ml) and sorbitol (0.1 mg/ml). In one flask was R-ethanol-1-^{3}H, 1-^{14}C; in the other sorbitol-2-^{3}H, 2-^{14}C. Utilizations of R-ethanol, sorbitol, ^{14}C, ^{3}H, and ^{3}H incorporation into lactate and water were determined. Ethanol utilization corresponded to that of ^{14}C from ethanol-1-^{14}C. Utilizations of ^{3}H were less than of ^{14}C, indicating isotopic effects. The ratio of ^{3}H into lactate to ^{3}H into water was usually somewhat less when ethanol rather than sorbitol was labeled. Between 5% and 25% of the ^{3}H of the sorbitol-2-^{3}H utilized was recovered in ethanol, but no ^{3}H from R-ethanol-1-^{3}H in sorbitol. From these data, that MEOS and catalase catalyze the removal of the R hydrogen as alcohol dehydrogenase (ADH), that ADH and sorbitol dehydrogenase are A-type enzymes, and assuming similar isotopic effects for MEOS, catalase and ADH, contributions (mean ± S.E.) to ethanol oxidation by MEOS and catalase (non-ADH paths) can be estimated.

SLICES	DIET	ETHANOL(mg/ml)	n	% Non-ADH PATHS
Rat	Stock	1	4	5.9 ± 7.2
Rat	Stock	3	7	13.9 ± 3.7
Rat	Ethanol	3	5	3.6 ± 6.1
Monkey	Stock	1	1	3.7
Monkey	Stock	3	4	25.9 ± 7.7

MEOS and catalase at most make only a small contribution (no more than 20%) to overall ethanol oxidation, except perhaps in monkey liver, under these *in vitro* conditions.